Antibiotic resistance in surface waters and biofilm-response to environmental contaminants

by

Keira Simonne Tucker

Dissertation presented for the degree of Doctor of Philosophy

Faculty of Science

Stellenbosch University

Supervisor: Prof. Gideon M. Wolfaardt
Department of Microbiology, Faculty of Science, Stellenbosch University,
Private Bag X1, Matieland, 7602, Stellenbosch, South Africa

Co-supervisors:
Dr. Marelize Botes
Department of Microbiology, Faculty of Science, Stellenbosch University,
Private Bag X1, Matieland, 7602, Stellenbosch, South Africa

Prof. Edward Feil
Department of Biology and Biochemistry, University of Bath,
Milner Centre for Evolution, Claverton Down, United Kingdom

December 2021

DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained herein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes three unpublished publications. The development and writing of the papers were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

Date: December 2021

Keira Tucker

Declaration from candidate

With regards to Chapters 3, 4 and 5, the nature and scope of my contribution were as follows:

Chapter 3

Nature of contribution	Extent of contribution
Planning and conducting of experiments, data analysis and manuscript writing	80%

The following co-authors have contributed to Chapter 3:

Name	Email and affiliation	Nature of contribution	Extent of contribution
Dr. Wendy Stone	Department of Microbiology Stellenbosch University	Assisted in experimental design, manuscript editing	15%
Prof. Gideon Wolfaardt	Department of Microbiology Stellenbosch University	Manuscript editing	4%
Dr. Marelize Botes	Department of Microbiology Stellenbosch University	Manuscript editing	1%
Prof. Edward Feil	Department of Biology and Biochemistry University of Bath	Manuscript editing	

Chapter 4

Nature of contribution	Extent of contribution
Planning and conducting of experiments, data analysis and manuscript writing	75%

The following co-authors have contributed to Chapter 4:

Name	Email and affiliation	Nature of contribution	Extent of contribution
Dr. Leonardos Mageiros	Department of Biology and Biochemistry University of Bath	Metagenomic analysis, manuscript editing	20%
Prof. Gideon Wolfaardt	Department of Microbiology Stellenbosch University	Manuscript editing	4%
Dr. Marelize Botes	Department of Microbiology Stellenbosch University	Manuscript editing	1%
Prof. Edward Feil	Department of Biology and Biochemistry University of Bath	Manuscript editing	
Prof. Barbara Kasprzyk-Hordern	Department of Chemistry University of Bath	Project co-ordinator and input into project proposal for funding	

Chapter 5

Nature of contribution	Extent of contribution
Planning and conducting of experiments, data analysis and manuscript writing	90%

The following co-authors have contributed to Chapter 5:

Name	Email and affiliation	Nature of contribution	Extent of contribution
Prof. Gideon Wolfaardt	Department of Microbiology Stellenbosch University	Manuscript editing	9%
Dr. Marelize Botes	Department of Microbiology Stellenbosch University	Manuscript editing	1%
Prof. Edward Feil	Department of Biology and Biochemistry University of Bath	Manuscript editing	

The above co-authors hereby confirm that:

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors,

2. no other authors contributed to respective chapters besides those specified above, and

3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in each chapter of this dissertation.

Declaration with signature in possession of candidate and supervisor.

ABSTRACT

Ensuring water security for the future has become important due to rapid urbanisation and diminishing freshwater resources. South Africa's water resources are scarce and as a result, reclamation of alternative freshwater resources such as treated wastewater is being investigated. There is growing evidence that drinking and wastewater treatment is either non-compliant to quality standards or lacking in certain communities. In areas with no infrastructure for wastewater removal, open sewers create a health risk for humans, animals, and the environment.

Poor antimicrobial stewardship, over-use and incorrect disposal has led to increased resistance to antibiotics, rendering some bacterial infections untreatable. There is a concern that sub-inhibitory concentrations of antibiotics create a selection pressure that promotes horizontal gene transfer and emergence of bacterial communities that are resistant to antibiotics. Antibiotics, antibiotic resistant bacteria (ARB), as well as other contaminants that have been shown to promote antimicrobial resistance (AMR) such as heavy metals, enter surface waters and wastewater treatment works (WWTW) in trace concentrations via multiple pathways. As a result, WWTW are deemed hotspots for the emergence and dissemination of AMR. In addition, environmental waters are home to various matrices, including biofilms that are especially problematic in a clinical setting due to their antibiotic resistant and persistent nature.

The research presented in this dissertation aimed to contribute to the knowledge surrounding the abundance of ARB in WWTW and surface waters in a South African context. Although ARB and antibiotic resistance genes (ARG) were detected in WWTW effluent, the abundance of both were reduced compared to the influent, suggesting that WWTW played a role in reducing AMR in receiving waters, while exposure to sub-inhibitory concentrations of antibiotics did not result in a significant change in the number of target ARG in isolates selected as representatives of a cultured population. This was emphasised in an expanded study that monitored various regions over a year. In addition, it was shown that surface waters, biofilms and sediments influenced by anthropogenic activities from residential and industrial sectors had higher prevalence of ARB compared to samples influenced by agricultural activity. Metagenomic analysis revealed that ARG relating to efflux pumps were the most common compared to those specific for target antibiotics. Due to heavy-metals and antibiotics being present in the environment in trace concentrations, exposure of mixed-community biofilms to sub-inhibitory concentrations of these contaminants was investigated. AMR in the biofilms did

not increase, but it was suggested that the sub-inhibitory exposure promoted the development of persistent mixed community biofilms.

Treatment interventions are crucial for removing pollutants and AMR already present in the environment. However, with due recognition of the complexity involved when considering humans, animals, the environment and a diverse pool of contaminants, this dissertation argues the need to expand the approach for mitigation of emergence or dissemination of AMR in the environment by incorporating greater emphasis on antibiotic stewardship, policies around antibiotic usage in all sectors, and overall public awareness.

OPSOMMING

Die versekering van watersekuriteit het belangrik geword vir die toekoms as gevolg van vinnige verstedeliking en ’n afname in varswaterbronne. Na aanleiding van beperkte waterbronne in Suid-Afrika, word die herwinning van alternatiewe waterbronne soos behandelde afvalwater ondersoek. Daar is toenemend bewyse dat die behandeling van drink- en afvalwater nie aan die kwaliteits riglyne voldoen nie, of hierdie geriewe bestaan nie in sekere gemeenskappe nie. In areas sonder infrastruktuur vir die behandeling en verwydering van afvalwater, skep oop rioolwater 'n gesondheidsrisiko vir mense, diere en die omgewing.

Swak antimikrobiese beheer, oorbenutting en onvoldoende verwydering van antibiotika, het gelei tot verhoogde antibiotika weerstand, wat sommige bakteriële infeksies onbehandelbaar maak. Daar is kommer dat sub-inhiberende konsentrasies van antibiotika 'n seleksiedruk skep wat horisontale geenoordrag bevorder, asook die ontstaan van antibiotika-weerstandige bakteriese (AWB) gemeenskappe. Afvalwaterbehandelingswerke (AWBW) word beskou as brandpunte vir die opkoms en verspreiding van antimikrobiese weerstand (AMW) as gevolg van antibiotika, AWB, en ander besoedelingstowwe soos swaarmetale wat AMW bevorder, wat in spoorkonsentrasies in oppervlakwater en AWBW beland via verskeie roetes. Daarbenewens, dra omgewingswater verskeie matrikse, onder andere biofilms wat veral problematies is in die kliniese veld vanweë hul antibiotika-weerstandige en hardnekkige aard.

Die doel van hierdie proefskrif se navorsing was om die kennis rondom die voorkoms van AWB in AWBW en oppervlakwater in 'n Suid-Afrikaanse konteks te bevorder. Alhoewel AWB- en antibiotika-weerstandstandige gene (AWG) in AWBW-afvalwater opgespoor is, is die teenwoordigheid van albei verminder in vergelyking met die ontvangswater, wat daarop dui dat AWBW 'n rol speel in die vermindering van AMW in ontvangswater. Terselfdetyd het blootstelling aan sub-inhiberende konsentrasies van antibiotika nie gelei het tot 'n beduidende verandering in die aantal teiken AWG in isolate wat gekies is as verteenwoordigers van laboratorium gekweekte gemeenskappe nie. Hierdie is benadruk in 'n uitgebreide studie wat verskillende streke oor 'n jaar gemonitor het. Daarbenewens het oppervlakwater, biofilms en sedimente wat deur antropogene aktiwiteite beïnvloed is deur residensiële en industriële sektore, 'n hoër voorkoms van AWB getoon in vergelyking met monsters wat deur landbou-aktiwiteite beïnvloed is. Metagenomiese analise het getoon dat gene wat verband hou met uitvloeipompe die mees algemene AWG was in vergelyking met dié wat vir spesifiek antibiotika geteiken was. As gevolg van die teenwoordigheid van spoorkonsentrasies van swaar metale en antibiotika in die omgewing, is die blootstelling van gemende-gemeenskap biofilms

aan sub-inhiberende konsentrasies van hierdie besoedelstowwe bestudeer. AMW in biofilms het nie verhoog nie, maar dit was voorgestel dat die sub-inhiberende blootstelling die ontwikkeling van hardnekkige gemengde gemeenskaps biofilms bevorder het.

Behandelingsintervensies is van kardinale belang vir die verwydering van besoedelingstowwe en AMW wat reeds in die omgewing voorkom. Met voldoende erkenning aan die kompleksiteit verbonde met mense, diere, die omgewing, en 'n diverse mengsel van kontaminante, benadruk hierdie proefskrif dat die voorkoms en verspreiding van AMW in die omgewing moet verlaag word deur groter klem te lê op antibiotika-bestuur beleid rakende die gebruik van antibiotika in alle sektore, asook algemene publieke bewustheid.

ACKNOWLEDGEMENTS

To my supervisor, Gideon Wolfaardt – I am truly grateful for your insightful contributions to my way of thinking and for allowing my own creativity to be explored. Thank you for the opportunities that you have provided, and for always emphasising the need for a work-life balance.

To my co-supervisors, Marelize Botes and Edward Feil – for your guidance, advice and contribution and editing manuscripts and dissertation chapters.

Leonardos Mageiros – for your contribution to the metagenomic work, for teaching me the basics, your advice in a personal capacity and always being willing to help.

Barbara Kazprzyk-Horden and the rest of the ReNEW team – for affording me the opportunity to be part of an interdisciplinary project, travel, and network. I have learnt an incredible amount both academically and personally by being a part of this team.

Elizabeth Holton – for hosting me in the UK and our conversation that ignited the idea for the heavy metal + antibiotic biofilm experiments.

Carla Louw and Tobi Louw – for providing veterinary antibiotic prescription data.

Prof Daan Nel at the Stellenbosch University Centre for Statistical Consultation - statistical analysis.

Elanna Bester and the rest of the Wolfaardt lab – for making me feel at home when I knew no one and for your all help along the way.

Edward Archer - for facilitating sampling permissions, proof reading and informal editing.

Mercia Volschenk at the City of Cape Town, and the Stellenbosch Municipality – for permitting sampling of wastewater treatment plants.

Stellenbosch University and the Department of Microbiology – for the use of facilities, infrastructure, and partial funding

Central Analytical Facilities – for sequencing DNA samples.

Casper Brink and Sporatec – for performing ARISA analysis.

National Research Foundation (Grant numbers: 118159 and 130527), Water Research Commission (Project No. K5/2733) and Global Challenges Research Fund (Grant No: EP/P028403/1) - for funding

Alno Carstens – Thank you for being a friend, coffee rants, for helping with sampling, good company on the long lab days that turned into nights, and for always being someone I could bounce ideas off.

Wendy Stone – No amount of thanks would be enough, but I'll try. Thank you for your assistance with qPCR and CEMS. For your thought provoking comments and input into my work, particularly Chapter 3. Thank you for being a friend, showing endless support and encouragement. For always being game for an adventure and for inspiring and motivating me daily. Thank you for always lending an ear when I needed a vent. I have learnt so much from you both academically and personally.

Deborah and Jacobus; Kirstie and Francois; and Marina and Steve - I am so lucky to have met you and been a part of your children's lives. They played such a pivotal role in my ability to keep going and brought me so much joy with games, dancing, music, and creativity. Thank you for letting me be a part of your families, for listening to my struggles and for always being interested in what I was doing in the lab.

My uncle, aunt and cousin, Brett, Elzet and little Shari Ayres – You have played such a huge role in my success. Thank you for always being an escape, your endless hospitality, for your support, empathy, gentle guidance, advice, and bringing so much joy, laughter, and positivity into these four years. You all inspire me to lead a life of gratitude and I am so thankful for everything you have done for me.

My sister and brother-in-law Chloe and Dayne Goetsch – Thank you for listening to my sometimes-never-ending explanations of what I do, probably not understanding a word, but being cheerleaders and rooting for me to achieve this goal anyway. For offering advice and helping me wherever you could.

My parents Debbie and Nigel Tucker - Thank you for paving the way and instilling a sense of curiosity in me that needs to see things through. Thank you for nurturing, guiding, and supporting me through the years and for listening to my frustrations and celebrating my triumphs. For guiding me through life's challenges and always being there for me.

To my fiancé, James Broughton – No words can thank you enough for your constant support, advice, love, reassurance, and motivation despite the distance between us. Your willingness to help with anything (mostly). Thank you for your patience, enduring the endless thesis talk and pushing me to keep going after pandemic-induced meltdowns. Thank you for everything.

CONTENTS

DECLARATION i
ABSTRACT iv
OPSOMMING vi
ACKNOWLEDGEMENTS viii
LIST OF ABBREVIATIONS xiii
LIST OF FIGURES xvi
LIST OF TABLES xix

CHAPTER 1:INTRODUCTION 1
1.1 Motivation for Study 1
1.2. Aims and Objectives 2

CHAPTER 2: REVIEW OF LITERATURE 4
2.1. History of antimicrobial resistance 4
2.2. Emergence of antimicrobial resistance 5
2.3. Mechanisms of resistance 6
2.4. Antibiotics of interest 8
2.4.1. Amoxicillin 8
2.4.2. Carbapenems 9
2.4.3. Gentamicin 9
2.4.4. Sulfamethoxazole 10
2.4.5. Colistin 10
2.5. Heavy metals: Zinc as an example 11
2.6. High-risk organisms 11
2.7. The role of environmental waters in AMR 14
2.7.1. Antibiotic contamination 16
2.7.2. Origin of resistant microorganisms 18
2.7.3. The role of biofilms and metal bioremediation and AMR 20
2.7.4. Presence of resistance genes 21
2.8. Final remarks 23

CHAPTER 3: WASTEWATER TREATMENT WORKS: A LAST LINE OF DEFENCE FOR PREVENTING ANTIBIOTIC RESISTANCE ENTRY INTO THE ENVIRONMENT 24
3.1. Introduction 25
3.2. Materials and methods 26
3.2.1. Sampling locations and procedure 26
3.2.2. Changes in antibiotic resistance during wastewater treatment 26
3.2.3. Sub-MIC impact on AMR 30
3.2.4. Statistical analysis 31
3.3. Results 32

3.3.1. Changes in AMR during wastewater treatment 32
3.3.2. Sub-MIC impact on AMR 36
3.4. Discussion 38
3.4.1. Changes in AMR during wastewater treatment 38
3.4.2. Sub-MIC impact on AMR 41
3.5. Conclusion 43
3.6. Acknowledgements 43
APPENDIX A 42

CHAPTER 4: EXPLORING ENVIRONMENTAL ANTIBIOTIC RESISTANCE IN AN URBAN HYDROLOGICAL CYCLE 51
4.1. Introduction 52
4.2. Materials and Methods 53
4.2.1. Sample sites and collection 53
4.2.2. Enrichment of highly resistant Gram-negative bacteria 55
4.2.3. Whole genome sequencing 55
4.2.4. Whole community analysis 56
4.2.5. Statistical Analysis 56
4.3. Results 57
4.3.1. Percentage resistance 57
4.3.2. Whole community and single colony genomics 60
4.4. Discussion 64
4.5. Conclusion 69
4.6. Acknowledgements 69
APPENDIX B 70

CHAPTER 5: SUB-MINIMUM INHIBITORY CONCENTRATIONS OF ZINC CHLORIDE AND SULFAMETHOXAZOLE-TRIMETHOPRIM PROMOTE PERSISTER CELL DEVELOPMENT IN BIOFILMS 85
5.1. Introduction 86
5.2. Materials and Methods 87
5.2.1. Sampling and inoculum preparation 87
5.2.2. Determination of MIC and MIC_{50} concentrations 87
5.2.3. Biofilm studies 88
5.2.4. Analysis of bacterial diversity 91
5.2.5. Microscopy 91
5.3. Results 92
5.3.1. Determination of MIC_{50} 92
5.3.2. Whole-community biofilm CO_2 profiles 92

5.3.3. SMX:TMP and $ZnCl_2$ MICs of viable planktonic and harvested biofilm cells 96
5.3.4. Analysis of bacterial diversity.....98
5.3.5. Microscopy.....99
5.4. Discussion.....101
5.5. Conclusion 107
5.6. Acknowledgements.....107
APPENDIX C 108

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS……...110

CHAPTER 7: REFERENCES…….... 115

LIST OF ABBREVIATIONS

AMR	Antimicrobial Resistance
AMX	Amoxicillin
ARB	Antibiotic resistant bacteria
ARG	Antibiotic-resistance genes
AS	Activated Sludge
AWaRe	Access, Watch and Reserve
CDC	Centres for Disease Control
CEMS	Carbon Dioxide Evolution Measurement System
CFU	Colony-forming Unit
CLSI	Clinical Laboratory Standards Institute
CSIR	Council for Scientific and Industrial Research
CST	Colistin
DWS	Department of Water and Sanitation
EPS	Extracellular polymeric substance
ESBL	Extended spectrum β-lactamase
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GD	Green Drop
GIT	Gastrointestinal tract
GM	Gentamicin
HGT	Horizontal gene transfer
HIV	Human Immunodeficiency Virus
KPC	*Klebsiella pneumoniae* carbapenemase

LMIC	Low- to Middle-Income Countries
LPS	Lipopolysaccharide
MAC	MacConkey
MDR	Multi-drug resistant
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentration
MIC_{50}	Concentration inhibiting 50% of isolates
MRSA	Methicillin Resistant *Staphylococcus aureus*
NCBI	Nucleotide Centre for Biotechnology Information
NDH	National Department of Health
NDM	New Delhi Metallo-β-lactamase
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
NMDS	Non-metric multi-dimensional scaling
OD	Optical density
OXA	Oxcillinase
PABA	*p*-aminobenzoic Acid
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RAS	Return activated sludge
SA	South Africa
SMX	Sulfamethoxazole

TB	Tuberculosis
TMP	Trimethoprim
TSB	Tryptic Soy Broth
UTI	Urinary tract infection
VBNC	Viable but non-culturable
WHO	World Health Organisation
WWTW	Wastewater treatment works
Zn	Zinc
$ZnCl_2$	Zinc chloride
ZnO	Zinc oxide

LIST OF FIGURES

Figure 2.1. Deaths attributable to antimicrobial resistance (AMR) each year by 2050 (O'Neill, 2014) 4

Figure 2.2. Timeline of the discovery of various antibiotics and the subsequent emergence of resistance to the respective classes (Alcock, 2018). 5

Figure 2.3. Percentage of susceptible and non-susceptible clinical isolates from 2018 and 2019 for meropenem (A) and gentamicin (B) against *A. baumannii*, gentamicin (C) and amoxicillin-clavulanic acid (D) against *K. pneumoniae,* and meropenem (E) and colistin (F) against *P. aeruginosa*. Adapted from NICD, 2019: https://mstrweb.nicd.ac.za/MicroStrategy/asp/Main.aspx 13

Figure 2.4. Green Drop Report scores (% compliance) for provinces in South Africa as indicated in the Department of Water and Sanitation Green Drop report of 2013 (Source: Department of Water Affairs, 2013). 15

Figure 2.5. Sources and pathways for the entry of microorganisms and antibiotics into the environment. 19

Figure 3.1. Percentage reduction of target antibiotic resistance genes from influent to effluent in two WWTW. Significant differences ($p<0.05$) in percentage reduction between WWTW, identified using a t-test, are shown by the asterisk (*). 32

Figure 3.2. Abundance of bacteria (OD_{600}) in the presence of amoxicillin (AMX-512 µg/ml), sulfamethoxazole (SMX-512 µg/ml), gentamicin (GEN-32 µg/ml) and colistin (CST-8 µg/ml) from influent, RAS and effluent samples in two WWTW (WWTW1-A and C, WWTW2-B and D) in sampling event 1 before (A-B) and after (C-D) being enriched in antibiotics. MacConkey media was used as a positive control (POS MAC) for colistin while Mueller Hinton Broth was used as a positive control (POS MHB) for the other antibiotics. Significant differences ($p<0.0.5$) between matrices are indicated by the black bar and asterisk (*). 33

Figure 3.3. Viable bacterial cells in the presence of gentamicin (GEN-32 µg/ml) and colistin (CST-8 µg/ml) from influent, RAS and effluent samples in two WWTW (WWTW1-A and C, WWTW2-B and D) in sampling event 2 before (A-B) and after (C-D) being enriched in antibiotics. Significant differences ($p<0.0.5$) between matrices are indicated by the black bar and asterisk (*). 34

Figure 3.4. Percentage of isolated species obtained from effluent samples that were previously exposed to antibiotics (n=19) and isolates obtained from various parts of the WWTW (n=11) (exploded slices). Isolates that indicate multiple species, had similar percentage identities to the query sequence when using BLAST. 35

Figure 3.5. Species composition from different WWTW effluent samples previously exposed to antibiotics (WWTW1: n=12, WWTW2: n=7), and not previously exposed to antibiotics (WWTW1: n=2, WWTW2: n=2) 35

Figure 3.6. MICs of whole communities in influent, RAS and effluent samples from two WWTW for amoxicillin (AMX), sulfamethoxazole (SMX), gentamicin (GM) and colistin (CST). The greater than symbol (>) indicates growth still occurred in the highest concentrations used and as a result the MIC is greater than the value depicted by the bar. 36

Figure 3.7. MICs of randomly selected isolates from WWTW, where each data point represents a single isolate. Mode MICs appear where data points cluster. Individual MICs, species identification and antibiotic exposure can be observed in Table S2.....37

Figure 3.8. Copy number of antibiotic resistance genes/16S rDNA copies in colonies isolated on antibiotic free media before and after sub-MIC exposure to sulfamethoxazole (*sul1* and *sul2*) and amoxicillin ($bla_{\text{KPC-1}}$). The line for each data set represents the median. 37

Figure 4.1. Summary of the urban water cycle. The conventional urban water cycle is depicted with blue arrows. Orange arrows indicate pathways that may occur in low middle-income countries due to lack of amenities. Multiple sources of water are used; lakes, dams and rivers contribute to surface water sources, while well points and boreholes give access to ground water. Adapted from Sowby, (2014).....52

Figure 4.2. Map showing Stellenbosch rivers and sample sites. 1- Eerste River, before influence of most sectors and used as a pristine site, 2- Plankenbrug river, upstream of informal settlement, 3- Plankenbrug river, downstream of informal settlement, 4- Plankenbrug river, industrial influence, 5- convergence of Eerste and Plankenbrug rivers, 6- wastewater treatment plant influent, 7- wastewater treatment plant effluent, 7a-point of treated effluent discharge, 8- wastewater effluent enters into Veldwagters river, 9- convergence of Eerste and Veldwagters rivers. For reporting purposes, sites 2, 3 and 4 are combined and referred to as “industrial and informal settlement” and sites 7 and 7a are combined as “wastewater effluent”. 54

Figure 4.3. Average percentage of antibiotic resistant bacteria (all antibiotics and matrices) at each site for each sampling event. 57

Figure 4.4. Percentage of Gram-negative bacteria that are resistant to carbapenems, colistin, gentamicin and sulfamethoxazole in aqueous, biofilm and sediment river sites, as well as wastewater influent and effluent. Each data point represents a sampling event with the median percentage denoted by the line. Horizontal red dotted lines indicate the range where most medians lie for all sites. 59

Figure 4.5. Whole community profiles in aqueous samples based on taxonomical class classification at selected sites and samples months. 60

Figure 4.6. Average number of specific, and multidrug ARG detected per isolate in pure cultures isolated on carbapenem media over all sampling events. 63

Figure 4.7. Number of target antibiotic resistance genes present at selected sites, identified from whole community aqueous samples.....64

Figure 5.1. CO_2 evolution measurement system (CEMS) set up for biofilm metabolism monitoring in real time. A) The reactor in its entirety, B) the CEMS component of the reactor

where the biofilm is cultivated, submerged in the water bath, and where dilution rates and retention times are calculated, and C) a schematic diagram of the CEMS principle.....88

Figure 5.2. Proportion of the growth of an overnight return activated sludge culture in different SMX:TMP and $ZnCl_2$ concentrations to determine their respective MIC_{50}s. For the SMX:TMP combination, the TMP concentration is plotted. The dotted red lines indicate the interpolated concentrations at which 50% of the population was inhibited. 92

Figure 5.3. Real time CO_2 production profiles and planktonic and biofilm viable cell counts from return activated sludge biofilms cultivated in a CO_2 Evolution Measurement System (CEMS). Biofilms were exposed to different treatment conditions selected based on the MIC_{50} values of the initial RAS culture, and from the planktonic cells identified during the experiment and are listed in Table 5.1. The CO_2 profile was primarily tracked in one reactor (black line), with intermittent comparisons between reactors (as described in section 5.2.3.2) indicated in green (repeat 1) and blue (repeat 2). Frames D-F are a repeated experiment of frames A-C. A, D – biofilm (i) (no initial treatment), B, E- biofilm (ii) (initial exposure to MIC_{50} of SMX:TMP), C, F – biofilm (iii) (initial exposure to MIC_{50} of $ZnCl_2$). The time points at which ARISA samples were taken for the repeated experiment are annotated on D-F. Malfunctioning of the laptop-interphase resulted in the loss of data between 127 h and 137 h seen in D - F. Planktonic plate counts were enumerated as CFU/ml sample collected from the reactor effluent, while biofilm biomass present in the silicone tubing was calculated to yield CFU/mm^2.....95

Figure 5.4. Median minimum inhibitory concentrations of biofilm and planktonic cell yield from cultivated biofilms after various treatments of SMX:TMP and $ZnCl_2$. Only the TMP concentration is shown on the left y-axis for the 19:1 SMX:TMP combination. D-F are repeats of A-C respectively. A, D – biofilm (i), B, E – biofilm (ii), C, F – biofilm (iii). Error bars indicate the maximum and minimum MICs from two repeats. 97

Figure 5.5. Alpha diversity indices indicating diversity and evenness of bacterial communities in planktonic and biofilm samples after each stage of the respective treatment conditions as described in Table 5.1. Higher values represent higher diversity and evenness.....98

Figure 5.6. Beta diversity of biofilm and planktonic communities after each stage of the respective treatment conditions as described in Table 5.1. Symbols of similar shape and colour are replicates. 99

Figure 5.7. BacLight stained planktonic cell yield from CEMS cultivated biofilms. A-C t = 66 h (upon removal of initial treatment), D-F t = 234 h (after recovery from 456:24 µg/ml SMX:TMP exposure). A and D – biofilm (i), B and E – biofilm (ii), C and F- biofilm (iii). Live cells are represented by green fluorescence and dead cells are represented by red fluorescence. 100

Figure 5.8. BacLight stained biofilm cultivated in CEMS. A-C – biofilm (i), D-F – biofilm (ii), G-I – biofilm (iii). A, D and G – biofilm at 99h (first steady state), B, E and H – biofilm at 190h (after 456:24 µg/ml SMX:TMP treatment), C, F, and I – biofilm at 285h (after 608:32 µg/ml SMX:TMP treatment). 101

LIST OF TABLES

Table 3.1. Sequences and references of primers and probes used in both probe-based and SYBR Green-based qPCR assays.....28

Table 4.1. Number of target antibiotic resistance genes in each genus identified.....61

Table 5.1. Concentrations of antimicrobials (µg/ml) added to 0.3 g/L TSB during experimentation for each biofilm growth condition. 90

CHAPTER 1

INTRODUCTION

1.1 Motivation for Study

Rapid urbanisation and population growth results in the need to meet the demand for vital resources. Arguably one of the most vital resources is water (Herbig, 2019). Not only does it sustain life, but also ensures health through proper hygiene and sanitation. Climate change and ever-growing populations make establishing future water security a necessity to maintain adequate health. However, in low-middle income countries, ensuring water security is a concern due to severe droughts, lack of infrastructure and maintenance of existing infrastructure that decrease water quality and as a result, decrease the health of the population (Visser, 2018). One of the ways in which water security can be ensured is by reusing treated wastewater in several applications including irrigation, industrial processes, and other non-potable uses, provided certain chemical and microbial standards are met (Fito and Van Hulle, 2020). However, in a South African setting, these standards are often not met, and freshwater resources are diminishing. In addition, a large proportion of the population is without wastewater removal and treatment infrastructure and as a result, communities are heavily dependent on environmental surface waters such as rivers. These rivers become heavily polluted due to contamination and pose a health risk for humans, animals, and the environment (Herbig, 2019). If water resources are to be reallocated and reuse is to become the norm in the near future, the safety of treated wastewater and surface waters needs to be established to ensure public health.

One of the concerns for public health is the emergence of antimicrobial resistance (AMR). Common bacterial infections have become untreatable in some cases due to the ability of bacteria to evolve and develop resistance mechanisms to various antibiotics. This occurs as a result of the over-use and misuse of antibiotics in both humans and animals (Singer et al., 2016; van Hoek et al., 2011; Ventola, 2015). The occurrence of these resistant infections is predicted to increase, with Africa anticipated to have the second-highest death toll by 2050 (O'Neill, 2014). In addition, heavy metals that occur naturally in the environment, and are used in a number of products and industrial processes have also been suggested to induce antibiotic resistance (Chen et al., 2019). Some bacteria have intrinsic resistance to antibiotics while others acquire resistance by receiving antibiotic resistance genes (ARG) from various bacterial species via mechanisms of gene transfer, which allows bacterial populations to adapt and survive when certain antibiotics are present (Guo et al., 2015). Due to increases in resistance

to commonly available antibiotics, combinations of two antibiotics with different mechanisms of action, that exert a synergistic effect against an infection have been implemented. In addition, bacteria can form biofilms, sessile bacterial communities, that can adhere to almost any surface (Sehar and Naz, 2016). Rocks, litter, and pipelines in the environment and medical devices such as catheters, pacemakers, prosthetic limbs, and even biological tissues can be adequate surfaces for biofilm colonisation (Bryers, 2008; Sahoo et al., 2015). Biofilms are well known to be highly resistant to antibiotics and other contaminants and abiotic factors due to their structure and the presence of extracellular polymeric substance (EPS) which prevents diffusion of a particular treatment throughout the biofilm. Biofilm infections that can be treated with antibiotics are often found to be recurring infections, leading to concerns for public health (Balcázar et al., 2015).

Multiple studies have suggested that WWTW are hotspots for the emergence of AMR due to the influx of ARB into these environments, as well as antibiotics in trace concentrations that have been suggested to supply a selective pressure to the bacteria in the WWTW (Guo et al., 2017; Hultman et al., 2018; Wellington et al., 2013). Due to the lack of water treatment infrastructure in South Africa (SA) described by Visser (2018), there is concern that not only may treated wastewater be below adequate water quality standards, but also surface waters that receive untreated waste from informal settlements, while surface runoff from agriculture may further pollute freshwater resources, making them unsafe to repurpose if there is risk of disseminating AMR.

With most studies focusing on the WWTW as reservoirs of AMR dissemination, the sectors contributing the influent of WWTW is often overlooked in their contribution to AMR in the environment. The work presented in this dissertation aims to add to environmental AMR surveillance in SA, elucidate which sectors might contribute to AMR dissemination in the environment and analyse water samples in addition to sediment and biofilm samples.

1.2. Aims and Objectives

The overall aims of this research were to evaluate the presence of AMR in environmental waters, and the effect of environmental contaminants on biofilm susceptibility to an antibiotic combination.

To achieve this, the following objectives were formulated:

1. To use culture-dependent methods to determine the abundance of amoxicillin, sulfamethoxazole, gentamicin and colistin resistant bacteria, as well as using qPCR to quantify selected ARG in the influent and effluent of two WWTW (Chapter 3).
2. To use culture-dependent methods to determine the percentage of Gram-negative bacteria resistant to carbapenems, sulfamethoxazole, gentamicin and colistin in water, sediment and biofilm samples obtained from rivers influenced by various sectors of society (Chapter 4).
3. To determine which bacterial genera are dominant in the samples and which ARG are present in each environment using Illumina sequencing to perform whole community profiling (Chapter 4).
4. To determine if sub-inhibitory concentrations of zinc chloride and sulfamethoxazole: trimethoprim promoted increased resistance to these treatments in a mixed-community biofilm using carbon dioxide production as an indication of biofilm metabolism (Chapter 5).
5. To determine if exposure to zinc chloride and sulfamethoxazole: trimethoprim result in community shifts within mixed community biofilms (Chapter 5).

This dissertation is comprised of six chapters. There are three experimental chapters that are presented as journal articles and are introduced and discussed individually with general conclusions and recommendations presented in Chapter 6. To understand the impact and the current situation of AMR specific to a South African environmental context, sampling occurred in two WWTW initially to determine the role of selected treatment facilities on the abundance of resistant bacteria and target ARG that entered receiving waters after being treated (Chapter 3). Due to the results obtained in this chapter, the study site was expanded to rivers influenced by various sectors of society. Here, profiling of resistant bacteria and ARG was also performed to determine if other anthropogenic, agricultural, or industrial sites were responsible for dissemination of AMR (Chapter 4). Due to contamination and high pollution levels found at these sites, the final experimental chapter added novelty to the dissertation by determining if the exposure of sub-inhibitory concentrations of a heavy metal and antibiotic to biofilms promoted emergence of AMR.

CHAPTER 2

REVIEW OF LITERATURE

2.1. History of antimicrobial resistance

Antibiotics have cured and prevented many life-threatening diseases and their use has been a fundamental part of public health since the mid-1900s (Ling et al., 2015). However, the threat of antimicrobial resistance (AMR) leading to a post-antibiotic era has long been of scientific interest. In some instances, the post-antibiotic era has already arrived, with pan-drug resistance being observed in clinical cases globally (Branswell, 2017; National Department of Health, 2016). The dangers of multi-drug resistant (MDR) bacteria or so-called 'superbugs', have been highlighted to the public in various news platforms to create awareness of this issue (Ismail, 2016; Pillay, 2017). Furthermore, AMR infection statistics suggest that 50 000 deaths are seen annually in Europe and the US, while 700 000 deaths are observed globally, each year (Audi et al., 2016; Branswell, 2017; Karlamangla, 2017; O'Neill, 2014). This number is predicted to reach 10 million annual deaths on a global scale by 2050, with 4.15 million of these deaths predicted to be attributed to Africa alone (Figure 2.1) (O'Neill, 2014).

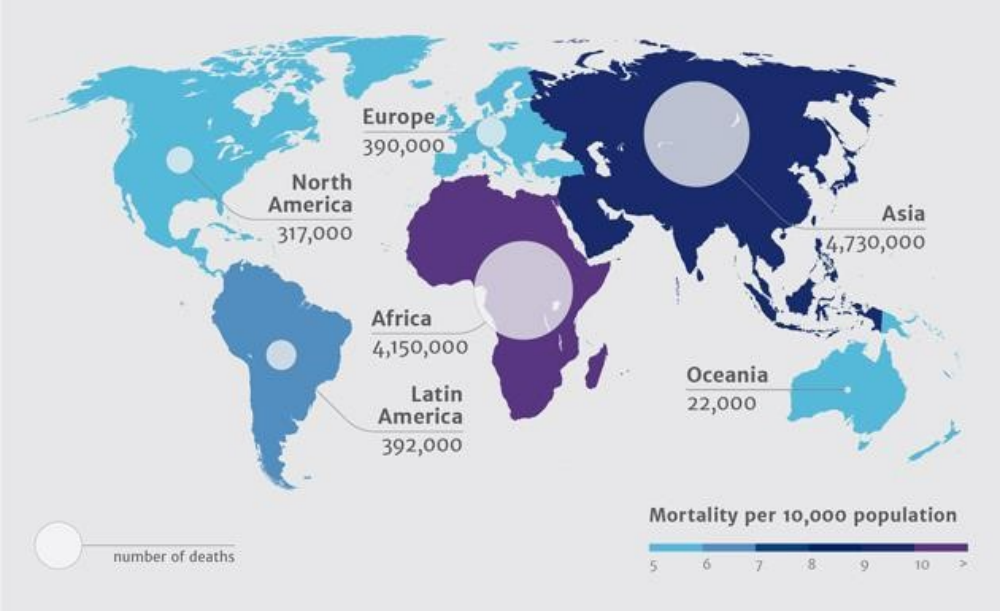

Figure 2.1. Deaths attributable to antimicrobial resistance (AMR) each year by 2050 (O'Neill, 2014)

While the discovery of penicillin in 1928 was a revolutionary event for the treatment of bacterial infectious diseases, its use in a clinical environment was not established until 1945. Despite this, resistance of *Staphylococcus pyogenes* to penicillin has been recorded as early as 1945 (Barber and Rozwadowska-Dowzenko, 1948). It has been found that in most cases,

resistance to an antibiotic develops rapidly after its clinical introduction. Figure 2.2 represents a timeline showing the development of resistance in relation to the discovery of the respective antibiotic (Alcock, 2018). Cephalosporins are an isolated case where resistance pre-dates the clinical use of the drug (Figure 2.2). Since many of the active ingredients of antibiotics are found in the environment, bacteria are often exposed to antibiotic-selective pressures and develop defence mechanisms to these compounds before the antibiotic is discovered and commercialised. As a result, the development of resistance to antimicrobial compounds that are used clinically is inevitable (Barber and Rozwadowska-Dowzenko, 1948; Laxminarayan et al., 2013).

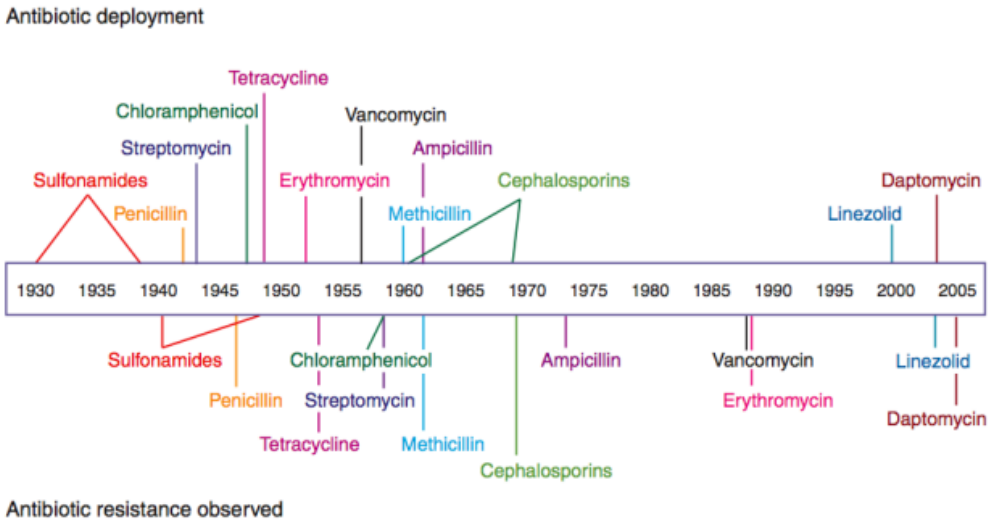

Figure 2.2. Timeline of the discovery of various antibiotics and the subsequent emergence of resistance to the respective classes (Alcock, 2018).

2.2. Emergence of antimicrobial resistance

The relationship between the use of antibiotics and the emergence of resistant bacteria is well known (Gelband et al., 2015). The over-prescription, non-compliance and misuse of antibiotics have contributed to the global AMR crisis. For example, the South African National Department of Health (NDH), as well as the US Centre for Disease Control (CDC) have estimated that 50% of all prescribed antibiotics for human consumption are unnecessary as a result of incorrect diagnoses (Centres for Disease Control and Prevention, 2013; National Department of Health, 2016). In addition to antibiotic use for human health, an overwhelming use of antibiotics in food animals/livestock has been observed (Centres for Disease Control and Prevention, 2013; van Hoek et al., 2011; Ventola, 2015). A review by Van Boeckel et al. (2017) showed approximately 131,000 tons of antibiotics were used worldwide in animal husbandry

in 2013. This is a substantially larger amount of antibiotic usage compared to human usage and is anticipated to reach 200 235 tons in the next decade (Van Boeckel et al., 2017).

In SA, 80% of antibiotics used are for agricultural, livestock, and domestic animal purposes (National Department of Health, 2016). The majority of these antibiotics are prescribed as preventative measures to promote growth. The quantities used often exceed therapeutic dosages (Laxminarayan et al., 2013). This emphasises the impact of veterinary antibiotic use on the development of AMR which, in turn, has a negative impact on human health. The connection between the use of antibiotics in animals and the development of AMR was recorded in 1969 in the Swann Report. In this report, the United Kingdom government suggested limiting the use of human antibiotics for prophylactic purposes in animals to prevent the emergence of resistance (Swann et al., 1969). As a result of this report, many countries in the European Union banned the use of antibiotics for growth promotion. However, it was not until 2006 that antibiotic use in livestock was banned completely in some areas (Laxminarayan et al., 2013). Heavy metals such as zinc and copper, have also been implemented into animal feed in conjunction with, or in place of antibiotics due to their growth promoting and antimicrobial properties (Ferreira Da Silva et al., 2007; Heo et al., 2013; Johanns et al., 2019; Yazdankhah et al., 2014). This practise is particularly dominant in pig farms (Aarestrup et al., 2002; Baker-Austin et al., 2006). As a result of the natural presence of heavy metals in the environment, and that they are not readily degraded, tolerance to heavy metals in bacteria has been observed. In addition to excessive antibiotic and heavy metal usage that selects for antibiotic resistant bacteria (ARB), the increase in global travel has also contributed to the rapid spread and proliferation of resistant bacteria across country boarders (Cantas et al., 2013).

2.3. Mechanisms of resistance

As with any living organisms, bacteria and other microorganisms evolve through genetic mutations as an adaptation to their changing surroundings. Such mutations are driven to benefit the microbe in relation to a particular habitat, including the development of defence mechanisms against antibiotics or other microbes that could secrete antimicrobial molecules as an attempt to out-compete other species (Read and Woods, 2014). Due to such adaptations, exposure to an antibiotic may drive the proliferation of the mutant organisms, while wild type organisms are killed, producing a bacterial population that is resistant to a specific antibiotic (Laxminarayan et al., 2013; National Department of Health, 2016; Read and Woods, 2014; van Hoek et al., 2011). While species evolution is a natural process, the rate at which resistance to antibiotics is developing is exorbitant due to anthropogenic influences. Many antibiotic

resistance genes (ARG) are located on chromosomal DNA and are thus passed on to each subsequent generation of the species, resulting in inherent or intrinsic resistance to a particular antibiotic (Essack et al., 2001). Alternatively, resistance genes can be acquired through horizontal gene transfer (HGT). This includes processes such as transduction, conjugation and transformation which allow plasmids and other mobile genetic elements to move to different bacterial species (broad host range) or be limited to one bacterial genus or species (narrow host range) (Schwartz et al., 2003; van Hoek et al., 2011)

ARG can encode several mechanisms of resistance to either single, or multiple antibiotics. Multiple genes can be responsible for resistance to a particular antibiotic, and it is possible for more than one resistance gene to be expressed simultaneously in a single cell (Duran et al., 2012). One of the best-researched resistance mechanisms is the production of the modification enzyme, β-lactamase. This enzyme renders penicillin antibiotics inactive by hydrolysing the β-lactam ring present in the antibiotic (Duran et al., 2012). Other mechanisms of resistance include altering cell wall permeability, preventing the antibiotic from entering the target site, and the presence of efflux pumps, which can actively remove the antibiotic from the microbial cell before it is able to exhibit its function. In addition, alternative metabolic pathways can be acquired by the cell that can bypass a crucial metabolic step which an antibiotic would normally inhibit. Enzymes usually targeted by an antimicrobial compound can be over produced, or the target site altered (Lin et al., 2015; van Hoek et al., 2011). On a molecular level, resistance to heavy metals in bacteria has been proposed to occur through sequestration, removal of the metal from the cell via efflux pumps, and detoxification of the metal (Bazzi et al., 2020).

It has been found that metal resistance genes and ARG can exist on the same transposable element and as such are co-transmissible (Bazzi et al., 2020; Cheng et al., 2019; Seiler and Berendonk, 2012). Due to the close proximity of these genes to one another, it is likely that they are expressed together (Seiler and Berendonk, 2012). As a result, bacteria exposed to heavy metals may develop tolerance and resistance to antibiotics as well. Cross-resistance can also occur where non-specific efflux pumps can export both metals and antibiotics out of a bacterial cell before the treatment can influence the cell (Bazzi et al., 2020). However, while some studies show increased heavy metal concentrations increase phenotypic antibiotic resistance (Berg et al., 2010; Knapp et al., 2011), others indicate the opposite is true (Hölzel et al., 2012; Stepanauskas et al., 2005). The conflicting findings may be a result of different heavy metal-antibiotic combinations being tested, with some metals potentially influencing the mechanism of resistance of the antibiotic (Seiler and Berendonk, 2012).

2.4. Antibiotics of interest

Countless antibiotics are considered a major concern for public health and are placed on the extensive list of "critical antimicrobials" by the World Health Organisation (WHO), reinforcing the severity of the AMR crisis (World Health Organisation, 2011). From this list, aminoglycosides, carbapenems, quinolones, β-lactams, macrolides, and multiple drugs used for tuberculosis treatment are among the antimicrobials which should receive high priority (World Health Organisation, 2011). In particular, the polymyxin antibiotic, colistin (CST), the penicillin-like compound, amoxicillin (AMX), carbapenems, and the aminoglycoside, gentamicin (GM) form part of the critically important antimicrobials, while the sulfonamide antibiotic, sulfamethoxazole (SMX) has been listed as highly important (World Health Organisation, 2011). AMX, GM and SMX are listed by the WHO's Access, Watch and Reserve (AWaRe) Index under the 'Access' group of antibiotics, which are first- or second choice empirical treatment for common or severe clinical syndromes and should always be available, while carbapenems and CST are listed in the "watch" and "reserve" groups, respectively (https://aware.essentialmeds.org/list) (World Health Organisation, n.d.).

2.4.1. Amoxicillin

Amoxicillin (AMX), a bactericidal β-lactam antibiotic that inhibits cell wall synthesis, is a semi-synthetic antibiotic which is effective against Gram-negative and Gram-positive infections as well as many anaerobic bacteria (Huang et al., 2011; Kaur et al., 2011). However, because of increased prevalence of extended spectrum β-lactamase (ESBL) producing bacteria, the β-lactamase inhibitor clavulanic acid has been regularly co-administered with AMX since the 1980s (Kaur et al., 2011). Such co-administration shows effectiveness as a first-line, broad-spectrum antibiotic for various common ailments such as respiratory- and dermal infections, tonsillitis, ear infections and many others (McIntosh, 2017). However, as stipulated by the South African Departments of Health, and Agriculture, Forestry and Fisheries report on the Antimicrobial Resistance National Strategy Framework for 2014-2024, one of the key drivers of AMR that needs to be addressed is the reliance of broad-spectrum antibiotics that will select for a wide range of resistant bacterial populations in comparison to more defined, narrow-spectrum antibiotics (National Department of Health, 2012). Moreover, other resistance profiles such as altered penicillin binding protein structures, reduced porin expression or increased efflux pump expression can also lead to resistance of AMX, even with β-lactamase inhibitor co-administration to combat specific AMR mechanisms (Bonomo, 2017). *AmpC*, bla_{TEM} bla_{SHV} are common ARG encountered for AMX resistance (Féria et al., 2002).

Unpublished antibiotic consumption data from both domestic and agricultural animals from veterinarians in the Stellenbosch region of South Africa also indicate frequent use of amoxicillin (Personal communication: Dr T. M. Louw and Dr C. Louw, Dept. of Chem Eng, Stellenbosch University).

2.4.2. Carbapenems

Like AMX, carbapenems are broad-spectrum, bactericidal, β-lactam antibiotics. They have been found to be effective against β-lactamase producing bacteria that would render other β-lactam antibiotics such as AMX and penicillin ineffective (Codjoe and Donkor, 2017). They have previously been last resort antibiotics however, due to the increased occurrence of resistance to cephalosporins, carbapenem use against Enterobacteriaceae has been increased (Papp-Wallace et al., 2011; Perez and Van Duin, 2013). As a result, resistance to carbapenems has emerged (Yang et al., 2016) and has also been observed in domesticated and wild animals (Köck et al., 2018). With the rise of carbapenem resistant bacteria, particularly in *Klebsiella pneumoniae* (*K. pneumoniae*) and other Enterobacteriaceae, the β-lactam class of antibiotics has become a major concern with a little under 1000 different β -lactamases being identified in the past two decades (Laxminarayan et al., 2013). In particular, those that produce carbapenemases that specifically inhibit carbapenem activity have disseminated globally (Perez and Van Duin, 2013). Of these, the New Delhi Metallo-β-lactamase (NDM), oxacillinases (OXA), Guiana extended-spectrum beta-lactamase (GES) and *K. pneumoniae* carbapenemase (KPC) are of most concern. These genes have also been identified in non-Enterobacteriaceae species which make infections with these organisms difficult to treat (Ebomah and Okoh, 2020).

2.4.3. Gentamicin

GM, an aminoglycoside antibiotic obtained from *Micromonospora purpura* in the 1960s, acts by binding to prokaryotic ribosomes, and as a result inhibits protein synthesis (Mingeot-Leclercq et al., 1999). Although GM is a broad-spectrum antibiotic, it is mostly used to treat infections caused by Gram- negative bacteria (Samadi et al., 2015; van Hoek et al., 2011). GM exhibits a large percentage of resistance in a number of bacterial species in SA (National Institute for Communicable Diseases, 2019). The main mechanisms of resistance are aminoglycoside modifying enzymes such as acetyltransferase, aminoglycoside phosphotransferase, and aminoglycoside nucleotidyl transferase. These enzymes are encoded by *aac*, *aph* and *ant* plasmid mediated gene variations respectively and alter the antibiotic through the adenylation, phosphorylation and acetylation of amine and hydroxyl groups (Duran

et al., 2012; Samadi et al., 2015; van Hoek et al., 2011). In addition, intrinsic mechanisms of resistance to aminoglycosides include efflux pumps, decreased permeability to the antibiotic and alteration of ribosomes (van Hoek et al., 2011).

2.4.4. Sulfamethoxazole

Being a member of the sulfonamide class of antibiotics, SMX acts by interfering with the biosynthetic metabolic pathway that produces folate in bacterial cells. Para-aminobenzoic acid (PABA) plays a vital role in this metabolic pathway as the enzyme, dihydropteroate synthase (DHPS) acts on it to produce folate. However, sulfonamides are structural analogues of PABA, thus competes with it and inhibits the enzyme activity of DHPS. As a result, folate is not produced and nucleic acid production is interrupted (van Hoek et al., 2011). However, resistance to these antibiotics occur through genetic mutations in the gene encoding DHPS which prevents sulfamethoxazole from binding to this enzyme (Suzuki et al., 2015). To combat this mechanism of resistance, SMX is prescribed together with trimethoprim (TMP) to create a bactericidal antimicrobial. This combination is used to treat a large variety of infections such as urinary tract infections (UTIs), *Nocardia* spp., *Toxoplasma* spp. and methicillin resistant *Staphylococcus aureus* (MRSA). In addition, HIV positive patients are treated with low doses of SMX:TMP to prevent acquiring opportunistic infections (Benson et al., 2009).

2.4.5. Colistin

Colistin (CST) falls into the polymyxin class of drugs, which are cationic cyclic polypeptides derived from *Paenibacillus polymyxa*. It is considered a last-resort treatment in the event of an otherwise multidrug resistant infection. It functions by targeting the negatively charged lipid A component of the lipopolysaccharide (LPS) found on the outer membrane of Gram-negative bacteria allowing diffusion of the polymyxin across the periplasmic space. As a result, pores form in the inner membrane and cellular components leak out of the cell resulting in cell death (Gao et al., 2016). Although its use in humans has been limited due to its renal- and neural toxic effects (Cai et al., 2012), the use of CST has been previously used extensively in livestock feed, especially in swine and poultry, as well as veterinary medicine. This has been identified as the root cause of the emergence of CST resistance which has been identified in a clinical setting (Gales et al., 2011; Newton-Foot et al., 2017; Rhouma et al., 2016). As a result, its use in livestock has been banned by the South African Veterinary Council (Gouws, 2015).

The use of CST for human treatment is slowly increasing as a result of the increase of bacteria which are resistant to commonly prescribed antimicrobials (Cai et al., 2012; Liu et al., 2016a).

As a result, CST is listed as an antibiotic requiring ongoing surveillance against pathogenic organisms such as *Acinetobacter baumannii (A. baumannii), K. pneumoniae, Escherichia coli (E. coli)* and *Pseudomonas aeruginosa (P. aeruginosa)* by the South African Department of Health (National Department of Health, 2016). The *mcr-1* gene was the first plasmid mediated CST resistance gene identified in China and as a result, interest in colistin resistance increased globally (Liu et al., 2016a). Since its discovery, numerous *mcr* genes (variants of *mcr-1*) have been identified in several Gram-negative organisms including *E. coli*, *Salmonella* spp. and *K. pneumoniae* in a number of countries and environments (Carroll et al., 2019; El Garch et al., 2017; Gao et al., 2016; Hu et al., 2016; Rhouma et al., 2016). The MCR-1 protein has been shown to aid resistance to CST by the addition of a phosphatidylethanolamine molecule to the Lipid A component of the lipopolysaccharide, thus reducing the affinity of Lipid A to CST (Liu et al., 2016a). The emergence of these plasmid-mediated resistance genes is concerning and puts into perspective the reality of the post-antibiotic era and reinforces the urgent need to prevent the emergence of full-blown resistance to this last resort antimicrobial and limit the spread of ARG to other pathogens.

2.5. Heavy metals: Zinc as an example

While many metals are toxic, others in low concentrations are vital for cell function. One of these is zinc (Zn) which serves as a cofactor for DNA synthesis and cell proliferation, in addition to being required for the activity of over 300 enzymes (Briffa et al., 2020). It has many anthropogenic uses including being present in paints, batteries, personal care products and cosmetics, and is used for galvanising steel (Briffa et al., 2020). Zn is also widely used as a feed supplement in post weaning piglets to prevent diarrhoea and to assist in growth promotion (Ghazisaeedi et al., 2020). It has been reported that the concentrations given greatly exceed the required dosage and as a result, excess Zn can be present in fertilizers if manure is used for that purpose, and subsequently enter the food chain, or surface runoff could result in Zn being washed into environmental waters (Bernhoft et al., 2014). As a result, Zn is a highly relevant environmental contaminant.

2.6. High-risk organisms

The Infectious Diseases Society of America has prioritised the most concerning pathogens in terms of AMR. Six organisms are collectively known as the ESKAPE organisms and include: *Enterococcus faecium* (*E, faecium*), *Staphylococcus aureus* (*S. aureus*), *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* spp. (Rice, 2008). These organisms are responsible for many infections and often contain genes encoding multiple mechanisms of resistance which

not only makes the infection more difficult to treat, but also prolongs the duration of illness for the patient, giving resistant bacteria more time to be transmitted to other individuals (Du et al., 2016; Laxminarayan et al., 2013).

In addition, the WHO released a priority pathogens list at the beginning of 2017. This list was established by several experts in a variety of fields and is split into three risk categories, namely critical, high, and medium (World Health Organisation, 2017). Carbapenemase producers such as *A. baumannii*, *P. aeruginosa,* E. *coli*, *K. pneumoniae* and other Enterobacteriaceae fall into the "critical" category since these organisms cause infections that are difficult to treat, and in some cases, are untreatable (Du et al., 2016; Laxminarayan et al., 2013; World Health Organisation, 2017). The "high-risk" category of the WHO priority pathogens list includes vancomycin resistant *E. faecium* and *S. aureus*, MRSA, clarithromycin resistant *Helicobacter pylori*, fluoroquinolone resistant *Campylobacter*, fluoroquinolone resistant *Salmonella* spp., and third generation cephalosporin and fluoroquinolone resistant *Neisseria gonorrhoeae*. Finally, the medium-risk priority organisms include penicillin-non-susceptible *Streptococcus pneumoniae*, ampicillin resistant *Haemophilus influenza* and fluoroquinolone resistant *Shigella* spp. (World Health Organisation, 2017).

In the US, Enterobacteriaceae resistance to carbapenems has increased by 1.4% in a decade (Laxminarayan et al., 2013). Seventy-one percent of *Klebsiella* spp. isolates and 50% of *E. coli* isolates obtained from neonates in developing countries were found to be resistant to GM, while 60-70% of *E. coli* isolates and close to 100% of *Klebsiella* spp. isolates were resistant to ampicillin (Zaidi et al., 2005). Staphylococcal resistance profiles against 10 different antibiotics showed that 87.9% of the clinical isolates were resistant to penicillin followed by 52.3% to ciprofloxacin. Thirty-two percent of the isolates were resistant to SMX:TMP, 28.9% to AMX-clavulanic acid and 31.5% to GM (Duran et al., 2012). From this, a high level of resistance to commonly used antibiotics is seen for staphylococcal isolates, reinforcing the WHOs listing of this genus as a high-priority organism (Duran et al., 2012; World Health Organisation, 2017). Extended-spectrum β-lactamase (ESBL) producers resulting in resistance to penicillin antibiotics have also given rise to concern (Wellington et al., 2013).

In SA, 67%-100% of *Enterococcus* spp. isolates from wastewater treatment works (WWTW) and hospital effluents in the Eastern Cape were resistant to a number of different antibiotics (Iweriebor et al., 2015). Many of the ESKAPE organisms and "critical" pathogens causing infections in SA are already resistant to the antibiotics of interest (Figure 2.3) (National Institute for Communicable Diseases, 2019). Even though differences are slight, in four of the six examples shown in Figure 2.3, the percentage of non-susceptible organisms increased in 2019 compared to 2018.

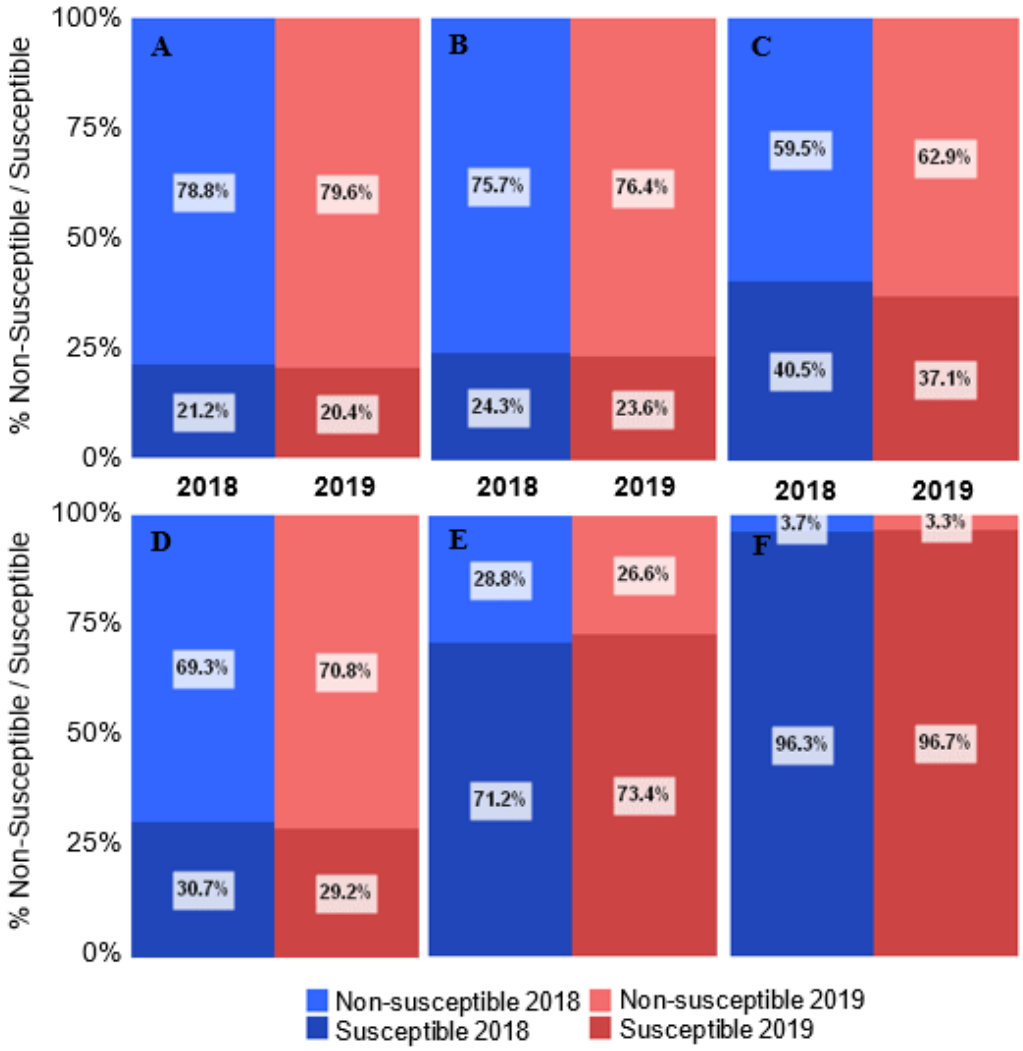

Figure 2.3. Percentage of susceptible and non-susceptible clinical isolates from 2018 and 2019 for meropenem (A) and gentamicin (B) against *A. baumannii*, gentamicin (C) and amoxicillin-clavulanic acid (D) against *K. pneumoniae,* and meropenem (E) and colistin (F) against *P. aeruginosa*.

Adapted from NICD, 2019: https://mstrweb.nicd.ac.za/MicroStrategy/asp/Main.aspx

2.7. The role of environmental waters in AMR

Rapid population growth and urbanisation have led to an increased demand for access to safe potable water resources. Furthermore, changes in annual weather patterns, especially for arid countries such as SA, which experiences cycles with low rainfall in most parts, emphasise the need for improved management of existing water resources, including more efficient re-use (Visser, 2018). To address this, SA has proposed the construction of a water reuse plant in their 2040 vision, where highly treated WWTW effluent will be blended with dam water, after which conventional water treatment will occur to meet international water quality standards. Reallocation of freshwater resources will become a norm in years to come with 8% of treated wastewater already being re-used in SA for in industrial processes and irrigation (City of Cape Town, n.d.).

However, ensuring water security in a developing country such as SA poses a major challenge and concern due to lack of proper hygiene and sanitation, as well as adequate infrastructure. For this reason, the Green Drop (GD) report was implemented in SA by the Department of Water and Sanitation (DWS) to audit municipal and private WWTW. Assessment criteria considered when compiling the report included the design capacity of the treatment plant, capacity exceedance, effluent quality referring to microbial, physical, and chemical compliance, and technical skills (management and operation) (Department of Water Affairs, 2013). Despite its implementation, the non-compliance of WWTW was evident, with only three of the nine provinces in SA showing wastewater treatment works that produced an effluent that was compliant with water quality standards. As a result, the GD system was discontinued, with the most recent available data being from 2013, shown in Figure 2.4 (Department of Water Affairs, 2013). The data showed that out of 824 WWTW assessed, only 199 were in the low-risk category, with 232 in high, 272 in medium and 121 in critical risk categories (Department of Water Affairs, 2013). Health concerns have resulted from the high pathogen loads released into surface waters, and further evidence of the range of pharmaceuticals and other micropollutants that may induce AMR (Berendonk et al., 2015).

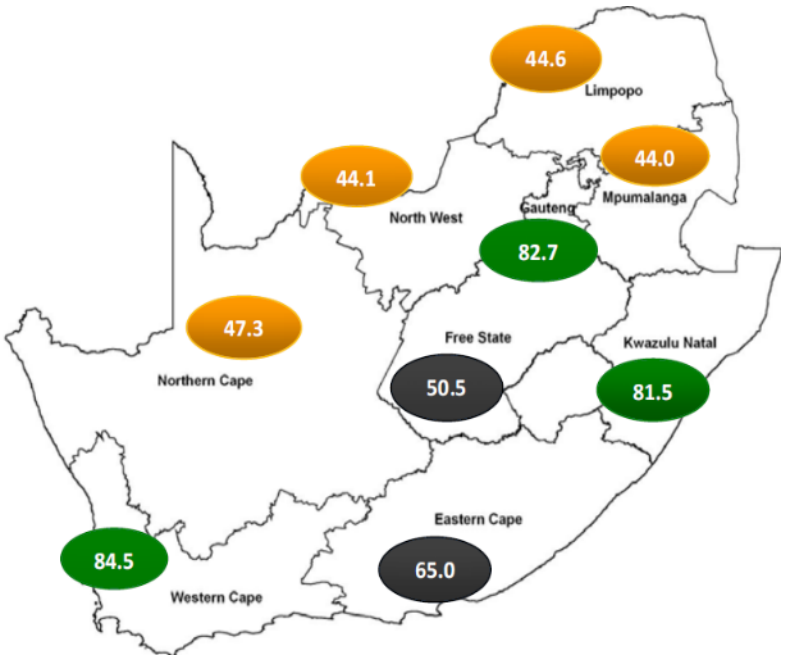

Figure 2.4. Green Drop Report scores (% compliance) for provinces in South Africa as indicated in the Department of Water and Sanitation Green Drop report of 2013 (Source: Department of Water Affairs, 2013).

Lack of infrastructure such as municipal sewage or greywater drainage systems or non-compliance of existing treatment facilities leads to the discharge of sewage and wastewater from daily use in informal settlements, which ends up in the proximity of households or communal areas, and eventually also in natural freshwater systems, contributing greatly to environmental pollution that enters surface waters including rivers (Greenberg et al., 2007; Harvey, 2018). In addition, healthcare and infection prevention control strategies are jeopardised which may play a role in AMR transmission (Knobler et al., 2003). As water safety and security is vital for the establishment and maintenance of public health, the presence of both high-risk pathogens and antimicrobials of concern in these environments needs to be established (Friedrich et al., 2009).

Although specified effluent standards should be adhered to, WWTW are not designed to eliminate all chemical and microbial contaminants, including antibiotics and pathogenic bacteria, which are often able to persist in the environment despite the treatment process. The microbial composition has been found to differ between influent and effluent, with a larger diversity of bacteria being present in the effluent opposed to the influent (Tang et al., 2016). A SA study indicated that pathogenic strains of *E. coli* present in wastewater effluent were resistant to multiple antibiotics, especially tetracycline (60.1%) and ampicillin (55.6%) (Adefisoye and Okoh, 2016). These ARB and ARG could accumulate in WWTW and promote

dissemination of AMR in WWTW effluent into environmental receiving waters as suggested by a number of authors who have labelled WWTW as hotspots for dissemination of AMR (Guo et al., 2017; Hultman et al., 2018; Lin et al., 2016; Madsen et al., 2012; Pazda et al., 2019; Pruden et al., 2012; Rizzo et al., 2013; Wellington et al., 2013).

2.7.1. Antibiotic contamination in WWTW

The presence of trace concentrations of antibiotics that might apply a selective pressure on bacterial communities in WWTW, resulting in proliferation of bacteria with resistance genes has led to WWTW being declared hotspots for AMR. Antibiotics can enter surface waters through various point- and non-point sources, mainly from anthropogenic activities depicted in Figure 2.5. In short, excretion from both human and animal (domestic and commercial sectors), as well as waste products from manufacturing and other industrial processes enter the sewerage system (Samadi et al., 2015; Wellington et al., 2013). These compounds can either be further metabolised in the sewage pipelines or transported in their parent forms at concentrations ranging from low (ng/L) to high (µg/L) levels. Polarity and solubility characteristics of antibiotics entering the wastewater treatment system determine the fate of the compound. Some compounds may be degraded while others enter rivers or adsorb to sludge or environmental biofilms depending on the individual partition coefficient (K_d) of the antibiotic (Wellington et al., 2013; Zhang and Li, 2011). Among the antibiotics most commonly found in wastewater are sulphonamides, fluoroquinolones and tetracyclines which are also seen to persist in downstream environments (Huang et al., 2011). This occurs due to these classes of antibiotics having stable chemistry; thus, degradation of these compounds is very slow. In contrast, antibiotics such as aminoglycosides and β-lactams are unstable, and therefore are not detected in environmental waters in high concentrations, despite their frequent use, as they degrade readily, however this does not mean they are completely absent from wastewater effluent (Kümmerer, 2009). Studies involving antibiotic presence in sludge, sediment and environmental biofilms are limited compared to those involving wastewater (Zhang and Li, 2011).

There is significant variation in concentrations of the same compound in WWTW (Archer et al., 2017; Petrie et al., 2015). This occurs because of the different sectors that feed into WWTW. For example, a WWTW that receives influent from hospitals and domestic sectors will have a different antibiotic profile to those that receive influent from industrial or agricultural sectors. In addition, different treatment facilities utilise different treatment

methods, which can lead to differences in the efficiency of the removal of contaminants. As WWTW effluent is discharged into downstream rivers and other surface waters, these bodies can play a role in emergence of AMR, as well and effect the overall health of the environment (Edokpayi et al., 2017).

Xu et al., 2015 showed that sulfonamides were only removed from sewerage treatment plants by 11.6 % and were found in concentrations ranging from 648.1 ng/L to 2518 ng/L between different treatment plants. In this group of antibiotics, SMX is often the most detected in WWTW (Zhang and Li, 2011). One of the few studies indicating antibiotic prevalence in SA environmental water focused on SMX concentrations in the KwaZulu-Natal province. These SMX concentrations were found to be 48.2 ng/L in rural surface waters, 2561 ng/L in urban surface waters and 3612 ng/L in WWTW effluent, suggesting that these antibiotics accumulate in WWTW (Suzuki et al., 2015). When compared to the Gauteng province in SA, SMX was found at an average of 1344.8 ng/L in wastewater effluent with a removal efficiency below 25% (Archer et al., 2017). These high concentrations, especially in an African setting make sense due to the high use of this antibiotic for treatment of bacterial infections in patients from countries where immunocompromising diseases such as HIV are rife (Suzuki et al., 2015). The median concentrations of SMX were also found to be amongst the highest out of several antibiotics detected in wastewater influents (250 ng/L), effluents (50 ng/L), and environmental water (8 ng/L) (Watkinson et al., 2009). Here it is evident that although SMX was not removed completely, the treatment process did assist in reducing the concentration of SMX that entered the environment. The highest concentration of SMX detected in WWTW influent from the literature consulted was 5597 ng/L (Peng et al., 2008), while that of the effluent was 6000 ng/L (Batt et al., 2006). Trimethoprim, a drug used in combination with SMX in a 1:20 ratio for optimal synergistic effect *in vivo*, is administered in a ratio of 1:5 to allow for a uniform concentration throughout blood and tissues of 1:20 (Smilack, 1999). TMP was also shown to persist in wastewater effluent from concentrations of 10 ng/L (Watkinson et al., 2009) to 3052 ng/L detected in the United Kingdom (Kasprzyk-Hordern et al., 2009). Trimethoprim was detected in SA wastewater effluent at a concentration of 1446 ng/L (Archer et al., 2017). These results show that there are substantial concentrations of this antibiotic in the WWTW effluent, which poses an environmental concern for AMR development.

Of the β-lactam class of antibiotics, oxacillin, cloxacillin, ampicillin, AMX, penicillin G and penicillin V are the most frequently detected in WWTW (Zhang and Li, 2011). Of these,

penicillin V was found in the highest concentration in WWTW influent (13800 ng/L) and effluent (2000 ng/L) while 1400 ng AMX was detected per litre influent in the same study (Watkinson et al., 2009). Quinolones are also frequently detected in WWTW in relatively high concentrations. The most prominent antibiotics from this group are ofloxicin, ciprofloxacin and norfloxacin. These antibiotics are widely used in numerous countries, and as a result are detected in WWTW worldwide (Zhang and Li, 2011). Quinolone concentrations in Asian WWTW ranged from 728.8 ng/L to 7870 ng/L (Minh et al., 2009; Xu et al., 2015). Of the macrolides, another of the antimicrobial groups of concern mentioned previously, is a metabolite of erythromycin, erythromycin-H2O, which was detected in the highest concentrations (10025 ng/L and 4330 ng/L) in influent and effluent respectively (Kasprzyk-Hordern et al., 2009). Few studies reported on the presence of CST in environmental waters. Due to the poor metabolism of the CST prodrug, colistin methanesulfonate, which is the treatment given to patients, only 20% is metabolised to active colistin sulfate (Labuschagne et al., 2016). The relatively high concentrations of some antibiotics in WWTW, in addition to the low removal efficiency of others allows for the application of a constant selective pressure to the microbiological communities present in the WWTW (Xu et al., 2015).

Antibiotics in wastewater are present in a large range of concentrations and differ within and between treatment plants, as well as between countries. There are multiple environmental and technical factors that may result in such variability. For instance, the timing of sampling, the sampling method and downstream processing of the samples may all lead to preferential detection of certain compounds. In addition, rain events can dilute the water and alter the concentrations in the treatment plant. It has also been noted that more commonly used antibiotics were detected in WWTW in higher concentrations (Zhang and Li, 2011). The sources of influent that each WWTW receives also influences the antibiotic concentrations which can play a major role in the development and dissemination of AMR. While there is extensive evidence of antibiotics being present in environmental waters, many of the ARGs detected in aquatic environments are novel discoveries (Alexander et al., 2020). Therefore, comparative sudies investigating antibiotic concentrations, ARGs and ARB in parallel in aquatic environments need to be performed to confirm potential correlations.

2.7.2. Origin of antibiotic resistant microorganisms

Activated sludge (AS), one of the most common treatment strategies in WWTW, utilises aerobic bacteria that form mobile biofilms (flocs) suspended in the mixed liquor to remove organic contaminants that can originate from industry, agriculture, and domestic sectors (Ruiz

et al., 2013). Microorganisms present in aeration tanks utilise compounds such as carbon, nitrogen and phosphorous in wastewater as nutrients to multiply, and as a result, remove the organic compounds from the wastewater. Although compounds are not completely removed, these microbial communities still play a major role in the treatment of wastewater to acceptable quality standards (Miralles-Cuevas et al., 2017; Nagwekar, 2014).

As microorganisms are ubiquitous, they enter the WWTW and surface waters through a variety of avenues seen for antibiotics in Figure 2.5. These include normal gut microbiota, as well as microbes excreted by patients with bacterial infections that subsequently enter WWTW through the sewage network, or surface waters if the infrastructure for waste removal is absent (Greenberg et al., 2007). Microbes used in industrial processes such as the mass production of enzymes or wine production, and environmental microorganisms or those present within agriculture (crops, livestock and in soils) can also be flushed into the WWTW and surface waters by rain or irrigation, in addition to occurring naturally in these environments. These microbes would add to those present in the WWTW that may already have mechanisms of resistance. Organisms receiving ARG can be both pathogenic and non-pathogenic. While the non-pathogenic ARB may seem less threatening, they may still have the potential to transfer resistance genes to pathogens (Samadi et al., 2015). Studies performed nearly two decades ago reported 40% of bacterial isolates from freshwater samples were resistant to more than one antibiotic (Ash et al., 2002). More recently, 92.7% of the isolates obtained from surface waters and sediments of an estuary were resistant to at least four classes of antibiotics (Eduardo-Correia et al., 2020).

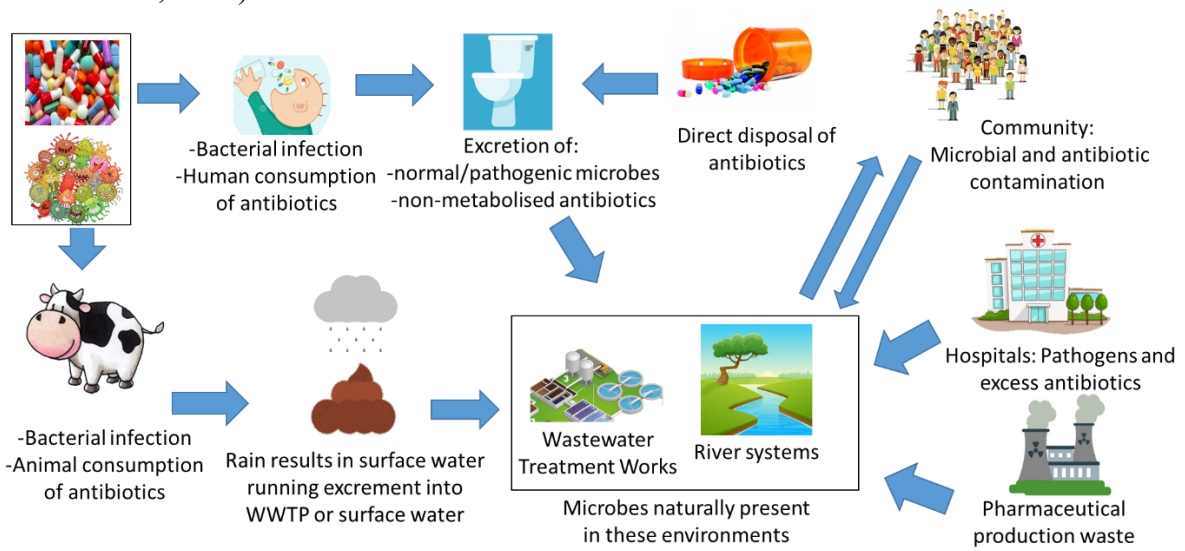

Figure 2.5. Sources and pathways for the entry of microorganisms and antibiotics into the environment.

Traditionally, to determine the prevalence of certain bacterial species or the bacterial composition of a sample, culturing techniques were used. While this provides an indication of the culturable microbes in an environment and is still representative of the sample, this typically accounts for less than 2% of the bacteria that are present in that environment (Wade, 2002). The remaining 98% are known as viable but non-culturable (VBNC) bacteria. Metagenomics is a powerful tool that enables the description of bacterial composition, including VBNCs, of a sample. As a result, the use of this tool for environmental microbiology is increasing. Proteobacteria have been found to be highly prevalent throughout the WWTW process and in effluent receiving waters, as shown in studies conducted in China and Finland (Guo et al., 2017; Hultman et al., 2018; Tang et al., 2016). This phylum includes Enterobacteriaceae, *Salmonella*, *Vibrio* and *Helicobacter* genera, among others. Many of these species are pathogens which are threats to public health and are listed as "critically important" by the WHO (World Health Organisation, 2017). Bacteroidetes and Firmicutes are phyla that are also observed in relatively high quantities (Guo et al., 2017; Hultman et al., 2018).

2.7.3. The role of biofilms and metal bioremediation in AMR

In addition to being present in mobile flocs in WWTW as mentioned above, biofilms are sessile communities of bacteria encapsulated in extracellular polymeric substance (EPS) that can occur naturally in the environment. They have been implemented in wastewater treatment plants for bioremediation purposes in the form of moving bed biofilm reactors, trickling filter bioreactors and activated sludge to name a few (Sehar and Naz, 2016). These communities can be problematic in the environment due to overgrowth in pipelines causing water quality issues (Sahoo et al., 2015). In addition, biofilms can also pose a problem in healthcare whereby opportunistic pathogens colonise surfaces within the body of on implants, catheters, prosthetic limbs, and other medical inserts (Bryers, 2008). As biofilms are notoriously more resistant to antibiotics compared to their planktonic counterparts, treatment of these infections becomes a challenge as antibiotics have little to no effect on biofilm formation resulting in persistent infections (Harrison and Turner, 2007; Sahoo et al., 2015). Biofilm formation limits penetration of antibiotics in the EPS to bacterial cells at the centre or base of the biofilm, enhancing their ability to persist in the environment (Balcázar et al., 2015). In addition, various microenvironments within the biofilm matrix may have different chemical compositions that may neutralise the antibiotic, rendering it ineffective (Sharma et al., 2019). The high cell density in biofilms can also provide an ideal environment for the transfer of ARG within, and between species in the case of a heterogenous biofilm when exposed to sub-inhibitory

concentrations of antibiotics (van Hoek et al., 2011). Some microorganisms in a planktonic state, such as *Pseudomonas* spp., utilise a biofilm as a defence mechanism when exposed to antibiotics and initiate biofilm formation (Sharma et al., 2019; Bryers, 2008), with sub-inhibitory concentrations of antibiotics leading to physiological changes in bacteria that increase the expression of various virulence factors and increase their colonisation of surfaces (Martínez, 2017). This may pose a threat for public health for AMR surface-associated infections. While sub-inhibitory concentrations of antibiotics do pose a threat to the emergence of AMR through genetic evolution including HGT, mutations, and recombination, physiological defense mechanisms against antibiotics are only evident while the sub-inhibitory selective pressure is applied (Martínez, 2017).

Due to the natural occurrence of heavy metals from the Earth's crust, as well as frequent use in industrial and agricultural applications, soil and water are at risk of becoming heavily polluted at toxic concentrations (Chen et al., 2019). Their bioaccumulation properties, that pose a risk to animal and human health and have the potential to have a detrimental effect on the food chain (Mishra et al., 2019). Common heavy metals include copper, aluminium, lead, manganese, cobalt, and iron to name a few that have extensive applications in industry and the built environment (Tchounwou et al., 2012). However, biofilms have been shown to increase production of exopolysaccharide (EPS) when exposed to high concentrations of heavy metals, leading to heavy metal tolerance, and as a result, biofilms can be used for bioremediation of these contaminants (Gurbanov and Severcan, 2020). Concentrations of Zn have been found to vary between countries and the environment in which it is found; water (18 - 35 µg/ml), sediment (50 - 267 mg/kg) and sewage sludge (408 - 1640 mg/kg) (Seiler and Berendonk, 2012). These concentrations are of concern as it has been shown that MRSA isolated from porcine and bovine samples contained a zinc resistance gene with majority of these isolates also having phenotypic Zn resistance (Cavaco et al., 2011).

2.7.4. Presence of antibiotic resistance genes

As the wastewater treatment process was not specifically designed to remove resistance genes, they often persist in the effluent which may have detrimental consequences on human, plant, and animal life. New emerging ARG conferring resistance to aminoglycosides, β-lactams and fluoroquinolones in clinical settings have been identified in wastewater effluent, indicating that genetic material from clinical microorganisms that enter the WWTW can be transferred to environmental bacteria (Szczepanowski et al., 2009). The most reported ARG found in wastewater are those that encode resistance to aminoglycosides, tetracyclines, sulfonamides

and β-lactams while many plasmid mediated resistance CST resistance genes have also been identified in the environment globally (Furlan et al., 2020; Hembach et al., 2017; Kneis et al., 2019; Wise et al., 2018).

Sulfonamide resistance genes are also frequently encountered in WWTW. Among the plasmid mediated sulfonamide resistance genes are *sul1*, *sul2* and *sul3*, which were first identified in the 1980s (van Hoek et al., 2011). In areas that have not been recently exposed to sulfonamide antibiotics, it has been found that *sul* genes, in addition to tetracycline resistance genes (*tet*) persist in sediment for long periods of time (Muziasari et al., 2014). This suggests that emergence of ARGs in the environment may be independent of the concentration of antibiotics present in these environments.

Sul1 and *sul2* are commonly detected in high frequencies in WWTW effluent however, *sul3*, appears to be less prominent (Czekalski et al., 2012; Hultman et al., 2018; Suzuki et al., 2015). Multiple studies have shown that *sul3* is present in microorganisms found in animals but not those found in humans (Guerra et al., 2003; Suzuki et al., 2015; Wu et al., 2010) and that *sul3* was not detected in bacteria that had been cultured, indicating this gene is more likely to occur in VBNC organisms (Suzuki et al., 2013). Information regarding antibiotics and their respective resistance genes in SA wastewaters is limited, with the study performed by Suzuki et al (2015) in Kwa-Zulu Natal being amongst the first of its kind. It was found that the copy numbers of *sul1* and *sul2* were similar, being 10^{-2} -10^{-1} per 16S copy number (Suzuki et al., 2015). A study conducted in Michigan, USA, showed a maximum of 2.33 x 10^4 ARG copies per ml of wastewater effluent was present while 4.32 x 10^9 ARG copies/g of biosolids were detected (Munir et al., 2011). Similarly, another study showed that copy numbers of carbapenem resistance genes ranged from 1.54 × 10^3 copies/ml to 2.14× 10^5 copies/ml in WWTW effluents opposed to biosolids where gene copy number were substantially higher and ranged from 6.51 × 10^9 copies/g to 6.18 × 10^{10} copies/g (Yang et al., 2016). In a third study, roughly 94% of β-lactam resistance genes were detected in AS while 85% of these genes were detected in WWTW effluent (Szczepanowski et al., 2009). The high ARG copy number in bio solids could influence the dissemination of ARG and antibiotic resistant bacteria (ARB) if the bio solids are repurposed for farming instead of being incinerated or taken to a landfill.

Despite there being a consensus that ARG quantities decrease from influent to effluent in WWTW, other studies suggest that there is very little or no change in certain ARG copy numbers the wastewater treatment process (Munir et al., 2011; Xu et al., 2015). bla_{KPC-2},

bla$_{GES-1}$, and *bla*$_{IMP-1}$ were detected, predominantly in *A. baumannii*, and *E. coli*, in WWTW effluent after chlorination (Yang et al., 2016). In addition, the study by Munir et al., (2011) showed that there was no significant decrease in either ARG or ARB before chlorination compared to after chlorination. Other research has suggested that chlorination selects for ARB and induces the emergence of ARG however, the underlying mechanisms are unknown (Murray et al., 1984). It has also been observed in some cases that resistance genes that do persist in the effluent, were present in different bacterial orders to what they were in the influent showing that HGT does indeed occur in WWTW (Hultman et al., 2018).

2.8. Final remarks

Clearly, multiple factors may influence AMR development in environmental waters. With the vast differences between anthropogenic practices between continents and countries (such as agriculture, socio-economic differences, and pharmaceutical use) it is vital for each region/country to establish the priority areas that need to be addressed for the control and mitigation of AMR.

CHAPTER 3

WASTEWATER TREATMENT WORKS: A LAST LINE OF DEFENCE FOR PREVENTING ANTIBIOTIC RESISTANCE ENTRY INTO THE ENVIRONMENT

Abstract

With their large, diverse microbial communities chronically exposed to sub-inhibitory antibiotic concentrations, wastewater treatment works (WWTW) have been deemed hotspots for the emergence and dissemination of antimicrobial resistance (AMR), with growing concern about the risk of transmission of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) into receiving surface waters. This study explored (1) the removal of ARG and ARB in local WWTW, (2) the effect of sub-inhibitory antimicrobial exposure on ARG copy numbers in pure cultures from WWTW, and (3) a comparison between two WWTW with different treatment configurations. For each WWTW, qPCR was performed to determine the removal of *mcr3*, *sul1*, *sul2* and bla_{KPC-1} during the treatment process, and culture methods were used to enumerate and identify ARB. Bacterial colonies isolated from effluent samples were identified by 16S rDNA sequencing and their respective minimum inhibitory concentrations (MIC) were determined. These were compared to the MICs of whole community samples from the influent, return activated sludge and effluent of each WWTW. Resistance genes were quantified in 11 isolated cultures before and after exposure to sub-MIC concentrations of target antibiotics. Although ARG and ARB were present in post-chlorination effluent, the numbers in both WWTW effluents were notably reduced compared to the influent. *Sul1* and *sul2* gene copies increased in cultures enriched in sub-MIC concentrations of sulfamethoxazole, while bla_{KPC-1} decreased after exposure to amoxicillin. It was concluded, within the parameters of this study, that WWTW assist in the removal of ARB and ARG, but that sub-inhibitory exposure to antimicrobial compounds has a varied effect on ARG copy number in pure cultures.

3.1. Introduction

Antibiotics have cured many life-threatening bacterial infections and are also used prophylactically prior to surgeries and in animal husbandry (Ling et al., 2015; Salkind and Rao, 2011). Their use has been a fundamental part of public health since the mid-1900s (Ling et al., 2015). However, AMR emerged as a global health-care crisis, resulting in numerous investigations into factors that promote AMR emergence in the environment. With population growth, urbanization, and the additional crisis of an increased demand for water, with diminished freshwater resources (Visser, 2018), wastewater reclamation for agricultural and industrial processes has become a necessity (Fito and Van Hulle, 2020; Palomo-Briones et al., 2016). Several studies have shown the recalcitrance of antibiotic compounds during wastewater treatment and have suggested that wastewater treatment works (WWTW) may promote the development and dissemination of AMR and associated resistance genes (Guo et al., 2017; Hultman et al., 2018; Pazda et al., 2019). Spreading of ARB into environments downstream of WWTW is an ecological concern, as well as a risk for entering the food chain through livestock drinking contaminated water, potentially containing AMR zoonotic pathogens (Caicedo et al., 2019; Sano et al., 2016). Crops being fertilised or irrigated with WWTW waste sludge and effluent water create another pathway for AMR into the food chain. A greater footprint of AMR in the ecosystem has significant consequences. Multi-drug resistant (MDR) and extensively drug resistant (XDR) diseases are becoming increasingly prevalent, with infections resistant to at least three antimicrobial compounds becoming increasingly common (Pérez-Rodríguez and Mercanoglu Taban, 2019). With MDR bacteria also being isolated from WWTW (Adefisoye and Okoh, 2016), the movement of AMR into the food chain has the potential to place significant pressure on the race to develop novel antimicrobial compounds.

WWTW are designed to remove a large variety of inorganic and organic matter. However, rapid urbanisation and densification necessitate upgrades to maintain an adequate standard of water treatment, which includes the elimination of microbes. These environments contain extremely diverse microbial communities due to high, varying nutrient loads and the usage of microbes for bioremediation in WWTW (Osunmakinde et al., 2019; Singh et al., 2006) in addition to the entry of microbes from external environments. AMR has been frequently detected in WWTW, and these sites have consequently been deemed hotspots for AMR dissemination (Hultman et al., 2018; Pazda et al., 2019). A range of treatment strategies have been developed to improve the efficiency by which microbial contaminants are removed. These include UV irradiation, ozonation and membrane filtration. However, due to the cost involved,

incorporation of these new technologies into existing WWTW is not always feasible (Fouz et al., 2020).

Except for studies applying metagenomics, most investigations on AMR in WWTW focus primarily on single species that have been isolated from WWTW, or indicator organisms such as *E. coli* (Ekwanzala et al., 2018; Fouz et al., 2020). However, less common bacterial species, and multi-species interactions, may influence the outcome of AMR in these diverse environments (Osunmakinde et al., 2019). With different population dynamics, locations and proximity to hospitals, agriculture or industrial settings, conditions for the effective removal of ARG and ARB may differ between WWTW. Therefore, treatment strategies that work for one location, may not work for another (Jasim, 2020). To address this, two different WWTW discharges in Cape Town, South Africa (SA) were selected to gain insight into the dissemination of AMR in specific contexts, to guide future solutions for preventing AMR transmission through WWTW. This study aimed to determine the degree to which bacterial resistance to four antibiotics (amoxicillin, gentamicin, sulfamethoxazole and colistin) and associated ARG (bla_{KPC-1}, bla_{OXA-48}, *sul1*, *sul2* and *mcr3*) persist within WWTW. The effect of sub-inhibitory antibiotic concentrations on resistance gene prevalence in resistant isolates from WWTW was also considered.

3.2. Materials and methods

3.2.1. Sampling locations and procedure

Wastewater grab samples were collected in 100 ml sterile Schott bottles from two WWTW located in Cape Town, South Africa from the influent (post grit screens), return activated sludge (RAS), and treated effluent (post-chlorination in WWTW1 and post-chlorination and post maturation pond in WWTW2). All samples were stored on ice during transport to the laboratory and stored at 4 °C until processed, which was within 2 h of arriving at the laboratory.

3.2.2. Changes in antibiotic resistance during wastewater treatment

3.2.2.1. Abundance of target ARG

ARG conferring resistance to amoxicillin (AMX; bla_{OXA-48}, bla_{KPC-1}), sulfamethoxazole (SMX; *sul1* and *sul2*) and colistin (CST; *mcr3*) were quantified with qPCR in influent, RAS and effluent WWTW samples.

3.2.2.1.1. DNA extraction and gene synthesis

DNA was extracted from 2 ml aliquots of the collected wastewater samples using the Quick-DNA Fecal/Soil Microbe Kit (ZymoResearch) according to manufacturer's specifications. This kit has inhibitor removal technology, which ensured that potential PCR inhibitors from the environmental samples were removed. Positive target gene controls were synthesised according to the sequences obtained from NCBI accession numbers (Appendix A, Table A1.1) that were flanked by the selected primers shown in Table 3.1. Synthesised genes were inserted into pBluescript II SK (+) cloning vector resulting in a 1498 bp plasmid. All genes, primers and probes were synthesised by Inqaba Biotech. Gene copies were normalised with 16S rDNA gene copies. The generation of standard curves and calculation of gene copy numbers are described in Appendix A (A1.1).

3.2.2.1.2. qPCR reactions and cycling conditions

sul1, sul2, mcr3 and 16s rDNA genes were quantified from WWTW samples using a SYBR green assay. Samples were run in duplicate and the reaction composition included the following final concentrations: 1× Luna® Universal qPCR Master Mix (New England Biolabs Inc.), 0.25 µM of each primer, 1 µl template DNA (concentrations in Appendix A, Table A1.3) and sterile milli-Q water to make the final reaction volume up to 20 µl. Cycling conditions for the SMX and CST resistance genes were according to the master mix manufacturer's instructions with 50 cycles of the 2-step amplification used. Cycling conditions for 16S rDNA involved an initial denaturation 94°C for 30 seconds, followed by 25 cycles of 94°C for 20 seconds, 58°C for 40 seconds, and 68°C for 90 seconds. A final extension at 68°C for 5 minutes was performed (Protocol adapted from manufacturers recommendations for OneTaq® 2× Master Mix with Standard Buffer, New England Biolabs Inc.).

The β-lactam (AMX) resistance genes were quantified using a probe-based assay containing 1× Luna® Universal Probe qPCR Master Mix (New England Biolabs Inc.), 0.4 µM of each primer, 0.2 µM probe, 1 µl template DNA and sterile milli-Q water to make the final reaction volume up to 20 µl. Three technical repeats and two biological repeats were performed. Cycling conditions were performed according to manufacturer's specifications.

Amplification was conducted using a LightCycler® 96 (Roche) system with excitation/emission wavelengths of 470/514 nm selected for the SYBR green reactions and FAM-labelled probe, and 577/620 nm selected for the CAL Fluor Red 610-labelled probe.

Negative controls containing sterile milliQ water instead of template DNA were included in all assays for each resistance gene.

Table 3.1. Sequences and references of primers and probes used in both probe-based and SYBR Green-based qPCR assays.

Probe-based assay					
Primer/probe name	**Sequence (5'-3')**	**Product Size**	**Probe label 5'**	**Probe label 3'**	**Reference**
bla_{OXA-48}f	GCGTGGTTAAGGATGAACAC	438			(van der Zee, 2014)
bla_{OXA-48}r	CATCAAGTTCAACCCAACCG				
bla_{OXA-48}p	AGCCATGCTGACCGAAGCCAATG		FAM	BHQ-1™	
bla_{KPC-1}f	TGCAGAGCCCAGTGTCAGTTT	138			(van der Zee, 2014)
bla_{KPC-1}r	CGCTCTATCGGCGATACCA				
bla_{KPC-1}p	TTCCGTCACGGCGCGCG		CAL Fluor Red 610	BHQ-2™	
SYBR Green-based assay					
Primer name	**Sequence (5'-3')**		**Probe label 5'**	**Probe label 3'**	**Reference**
Mcr3f	ACCTCCAGCGTGAGATTGTTCCA				(Li et al., 2017)
Mcr3r	GCGGTTTCACCAACGACCAGAA	169			
qSUL653f	CCGTTGGCCTTCCTGTAAAG				(Heuer and Smalla, 2007)
qSUL719r	TTGCCGATCGCGTGAAGT				
		67			
q*SUL2*_595f	CGGCTGCGCTTCGATT				(Heuer et al., 2008)
q*SUL2*_654r	CGCGCGCAGAAAGGATT				
		60			
16s rDNA 8F	AGAGTTTGATCCTGGCTCAG				(Felske et al., 1998)
16s rDNA 1512R	GTGAAGCTTACGGTTAGCTTGT TACGACTT				

3.2.2.2. Abundance of ARB

Antibiotic resistant bacteria were enumerated, and representative colonies were isolated and identified from two WWTW using two different methods to reduce potential culture bias and obtain a more diverse range of isolates.

3.2.2.2.1. Determination of antibiotic concentrations for ARB selection

To classify a particular organism as antibiotic resistant, its MIC to a particular antibiotic needs to be higher than the resistance breakpoint concentration set by the Clinical and Laboratory Standards Institute (CLSI, 2015) and European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2017). If the MIC is lower than the breakpoint, the organism would be deemed susceptible to that antibiotic. As a result, the CLSI and EUCAST resistance breakpoints were used to select starting concentrations of the selected antibiotics to incorporate into culture media for the selection of antibiotic resistant bacteria. As these documents obtain their values from pure cultures (i.e., laboratory conditions/strains), there was a need to determine MICs for environmental whole communities in order to select for highly resistant

organisms. A microbroth dilution susceptibility assay was performed in 96 well microtiter plates (Sigma Aldrich). As colistin (CST) is only active against Gram-negative bacteria, MacConkey (MAC) broth (Merck) (selective for Gram-negative bacteria) was used, while Mueller Hinton Broth (MHB) (Merck) was used for the other broad-spectrum antibiotics. Antibiotic concentrations were sequentially doubled as described by Andrews (2001), for colistin sulfate (CST; 1 -16 µg/ml), gentamicin (GM, 4-64 µg/ml), amoxicillin (AMX; 32 -512 µg/ml) and sulfamethoxazole (SMX; 64-1024 µg/ml). RAS samples obtained from the two WWTW were diluted in phosphate buffered saline (PBS) to obtain a 0.5 McFarland standard turbidity equivalent and used as the inoculum for the susceptibility testing. After incubation at 37°C for 18-24 h with shaking, optical density was measured at 600 nm, and $OD_{600} \leq 0.02$ indicated the MIC. Antibiotic concentrations that were two-fold lower than the determined RAS MICs (shown in Appendix A, Figure A2.1) were selected as the sub-MIC concentrations for the enumeration and selection of antibiotic resistant bacteria in WWTW.

3.2.2.2.2. Culturing, enumeration, and isolation

Five ml of the homogenised water samples from the WWTW influent, RAS, and effluent, respectively, were treated with 0.001% cycloheximide (w/v) to inhibit the growth of fungi and were subsequently serially diluted (1:10) with PBS to dilute solid matter in the raw samples. A droplet (20 µl) of each dilution was inoculated in duplicate into 96-well microtiter plates (Sigma Aldrich) containing MAC broth supplemented with 8 µg/ml CST, and MHB supplemented in individual wells with 32 µg/ml GM, 512 µg/ml AMX and 512 µg/ml SMX, respectively. Positive controls containing only MAC and MHB media with no antibiotic supplementation were also included. After 18-24 hours of incubation at 37 °C, optical density was measured at 600 nm (Biorad XMark Microplate Absorbance Spectrophotometer). Twenty µl of the respective cultures obtained in the 10^{-1} dilution were sub-cultured overnight in fresh broth containing the same antibiotic conditions for enrichment, a total of three times, after which serial dilutions were enumerated on agar plates containing the same antibiotic concentrations and incubated for 18-24 hours at 37°C. Four single colonies from each antibiotic plate were selected at random and streaked to purity. They were subsequently cultured in Tryptic Soy Broth (TSB) overnight and DNA was extracted using a method adapted from Crouse and Amorese (1987; protocol in Appendix A1.2) and stored at -20°C, with long-term storage at -80°C (40% glycerol). DNA concentrations for individual isolates are shown in Table A.1.

The abundance of antibiotic resistant bacteria in the WWTW effluent was lower than in the influent, in contrast to findings in previous studies, which indicated that WWTW promoted the dissemination of AMR (Adefisoye and Okoh, 2016; Czekalski et al., 2012; Rizzo et al., 2013). Therefore, a second sampling event was conducted to test the validity of this unexpected result. Samples were collected and processed as previously described however, only CST and GM were supplemented into MAC and MHB respectively to simplify the experiment. In addition to broth enrichment, samples were spot plated onto respective antibiotic plates before and after enrichment and incubated for 18-24 hours at 37°C to obtain plate counts. AMX and SMX were omitted here to simplify the experiment. Colonies from all plates before and after enrichment were selected at random and DNA was extracted from each pure colony and stored as described above.

3.2.2.2.3. Identification

The DNA extracted from each isolate was thawed and samples with a high DNA yield were diluted with TE buffer. PCR targeting the 16S rDNA gene was performed using universal primers 8F and 1512R (Table 3.1). Reaction components were combined to obtain final concentrations of 1.25 units OneTaq Polymerase (New England Biolabs), 200 µM dNTPs, 0.2 µM of each primer mentioned above, 1× OneTaq Buffer, 4 µl DNA template (concentrations in Appendix A, Table A1.3) and sterile milliQ in a 50 µl volume. Cycling conditions were as described previously for the qPCR reactions. Amplicons were sequenced (3730XL DNA Analyser; Applied Biosystems) and sequence chromatograms were trimmed and consensus sequences for each primer pair were constructed using BioEdit (v 7.05). BLASTn was performed for each sequence with the database selected for 16S ribosomal RNA sequences (bacteria and archaea). The species of isolates were identified based on query cover and identity using a threshold of 99%.

3.2.3. Sub-MIC impact on AMR

Whole community and pure culture MICs were determined to identify the effect of antibiotic exposure in sub-MIC concentrations on the copy number of ARG present in isolates obtained from WWTW.

3.2.3.1. MIC determination for pure cultures and whole communities

Whole-community and pure culture MICs were established. The 30 glycerol stocks previously isolated were revived in TSB overnight at 37°C. Susceptibility testing was performed in duplicate by microbroth dilution as described above, however the doubling dilution antibiotic

concentration ranges used were increased to accommodate highly resistant isolates; CST; 1-512 µg/ml, GM; 2-1024 µg/ml and AMX and SMX; 32-16384 µg/ml. After overnight incubation at 37°C, culture from wells containing growth at the highest concentration before inhibition (sub-MIC) was sub-cultured overnight at 37°C in TSB. DNA was extracted from these cultures as above and stored at -20°C.

For whole communities, to avoid initial culturing bias, the collected wastewater samples were used as inoculum. The conventional 0.5 McFarland standard turbidity could not be used to standardise inoculum density in wastewater samples due to the effluent samples having lower turbidity than required, and a large volume would have been required for filtration to concentrate the samples. Inoculum density was made uniform across all wastewater samples (influent, RAS, and effluent), by measuring the OD_{600} of the effluent (cleanest sample) and diluting the other samples with PBS to obtain a similar OD_{600} (0.018 ±0.015). Microbroth dilutions were performed as for the single colonies. In addition, DNA from influent, RAS and effluent samples was extracted and stored at -20°C.

3.2.3.2. qPCR measuring the effect of Sub-MIC concentrations on ARG

Sul1, sul2 and bla_{KPC-1} were quantified in pure cultures isolated during the second sampling event prior to antibiotic exposure, and after sub-MIC antibiotic exposure during the MIC experiments. Quantification was performed with qPCR as described previously to determine gene copy numbers. Generation of standard curves is described and shown in Appendix A.

3.2.4. Statistical analysis

Data obtained from culturing was analysed in GraphPad Prism (v. 6) using a multiple t-test to determine significance between the two WWTW in influent, RAS, and effluent samples. Student's T-tests for independent means were used to determine significant differences in resistance gene copy numbers between WWTW and in single colonies before and after exposure to sub-MIC concentrations of antibiotics. Significance was determined at a confidence interval of 95% ($p < 0.05$).

3.3. Results

3.3.1. Changes in AMR during wastewater treatment

3.3.1.1. Abundance of target ARG

The percentage reduction of target resistance genes between influent and effluent ranged from 69 to 100% as shown in Figure 3.1. Percentage reductions were higher in WTWW2 effluent compared to WWTW1 for three out of the four target genes, with more than a 99% decrease of *mcr3, sul1 and sul2*. *Mcr3* was completely removed from the effluent of WWTW2. The negative percentage reduction observed for bla_{KPC-1} in WWTW2 indicates an 80.9% increase in gene copies from influent to effluent. All samples were negative for bla_{OXA-48} genes (potentially sue to primer/amplification inefficiencies) and were therefore excluded. Copy numbers for each resistance gene are shown in Appendix A, Figure A2.2.

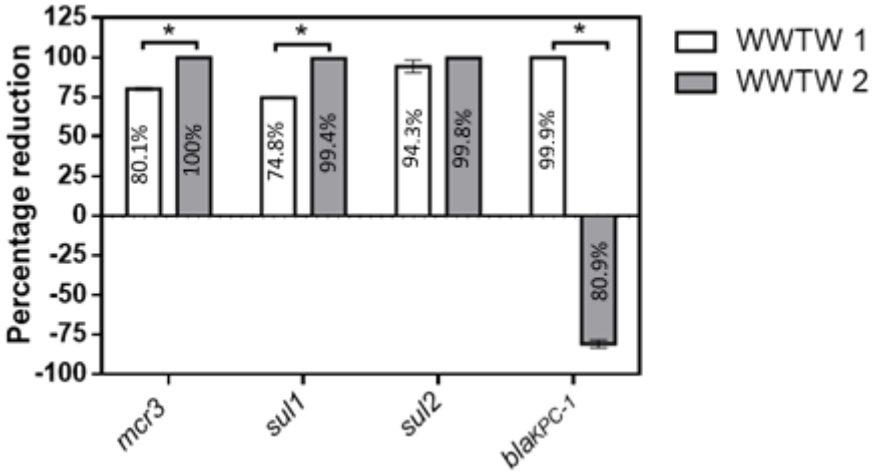

Figure 3.1. Percentage reduction of target antibiotic resistance genes from influent to effluent in two WWTW. Significant differences ($p<0.05$) in percentage reduction between WWTW, identified using a t-test, are shown by the asterisk (*).

3.3.1.2. Abundance of ARB

The abundance of highly resistant bacteria was similar for all antibiotics within each sample matrix, except for SMX, which had a visibly greater effect on the bacteria within the samples obtained from sampling event 1 (Figure 3.2A and B). The abundance of ARB in both WWTW effluents (Figure 3.2A and B) was lower compared to the influent and RAS, with the abundance of bacteria resistant to AMX, GM and CST in WWTW2 effluent lower than in WWTW1 (Figure 3.2 A and B). Cell numbers were also lower in sampling event 2 (Figure 3.3A-B) where CST was supplemented into the media. The number of GM resistant bacteria did not change in sampling event 2 between matrices or WWTW (Figure 3.3A-B). Upon enrichment in antibiotics, the abundance of ARB to all antibiotics increased in both sampling event 1 (Figure 3.2C-D) and 2 (Figure 3.3C-D). Most samples enriched in MAC and CST resulted in higher

abundance (1.5-2.2) in WWTW1 compared to WWTW2 (0.5-1.8) (Figure 3.2 C and D respectively). A higher OD_{600} in MAC broth compared to MHB was observed for most of the sample matrices (Figure 3.2A-D).

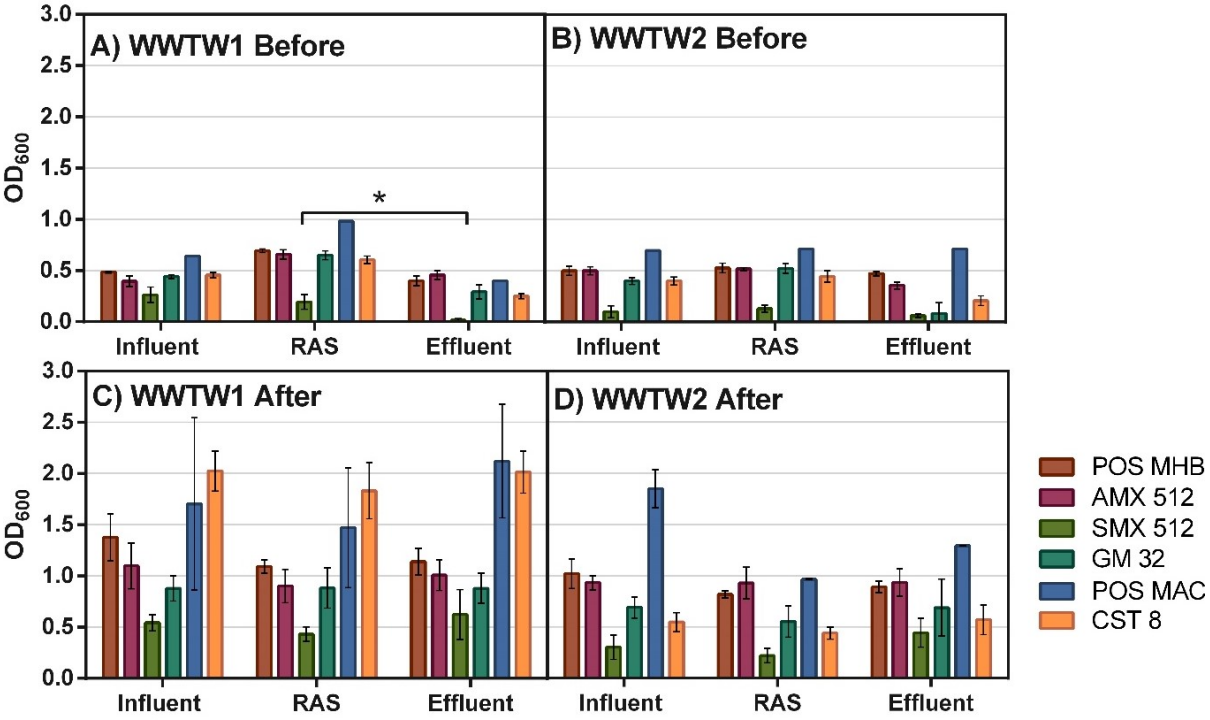

Figure 3.2. Abundance of bacteria (OD_{600}) in the presence of amoxicillin (AMX-512 µg/ml), sulfamethoxazole (SMX-512 µg/ml), gentamicin (GEN-32 µg/ml) and colistin (CST-8 µg/ml) from influent, RAS and effluent samples in two WWTW (WWTW1-A and C, WWTW2-B and D) in sampling event 1 before (A-B) and after (C-D) being enriched in antibiotics. MacConkey media was used as a positive control (POS MAC) for colistin while Mueller Hinton Broth was used as a positive control (POS MHB) for the other antibiotics. Significant differences ($p<0.0.5$) between matrices are indicated by the black bar and asterisk (*).

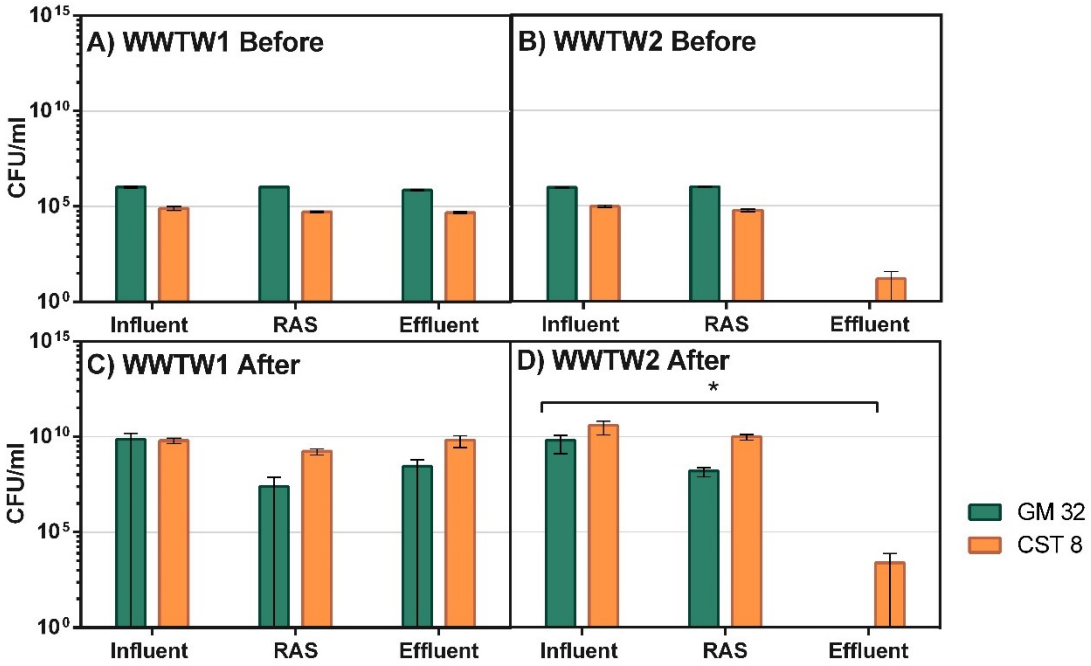

Figure 3.3. Viable bacterial cells in the presence of gentamicin (GEN-32 µg/ml) and colistin (CST-8 µg/ml) from influent, RAS, and effluent samples in two WWTW (WWTW1-A and C, WWTW2-B and D) in sampling event 2 before (A-B) and after (C-D) being enriched in antibiotics. Significant differences ($p<0.0.5$) between matrices are indicated by the black bar and asterisk (*).

Of the randomly selected single colonies that were isolated from both sampling events (n=30), *Morganella morganii* was the most dominant (20%), followed by *Escherichia fergusonii/Shigella flexneri* (13.3%), while *Citrobacter freundii, Pseudomonas indoloxydans, Klebsiella pneumoniae, Klebsiella quasipneumoniae* and *Enterobacter tabaci* each contributed 6.7% to the composition of the selected isolates. The remaining isolates each make up 3.3% of the sample (Figure 3.4). Figure 3.4 shows that there was a notable shift in community composition after enrichment in antibiotics.

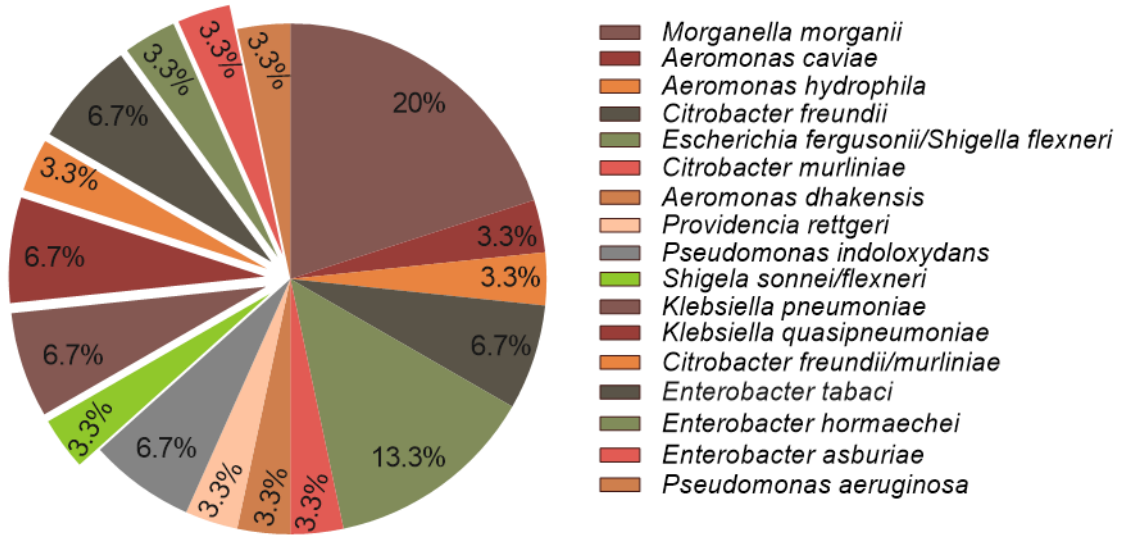

Figure 3.4. Percentage of isolated species obtained from effluent samples that were previously exposed to antibiotics (n=19) and isolates obtained from various parts of the WWTW (n=11) (exploded slices). Isolates that indicate multiple species, had similar percentage identities to the query sequence when using BLAST and could not be identified to the species level.

Species composition varied between WWTW as seen in Figure 3.5. Of the randomly surveyed species isolated before and after antibiotic exposure, *Morganella morganii* was a dominant species in both WWTW, with *Escherichia fergusonii/Shigella flexneri* also dominant in WWTW1.

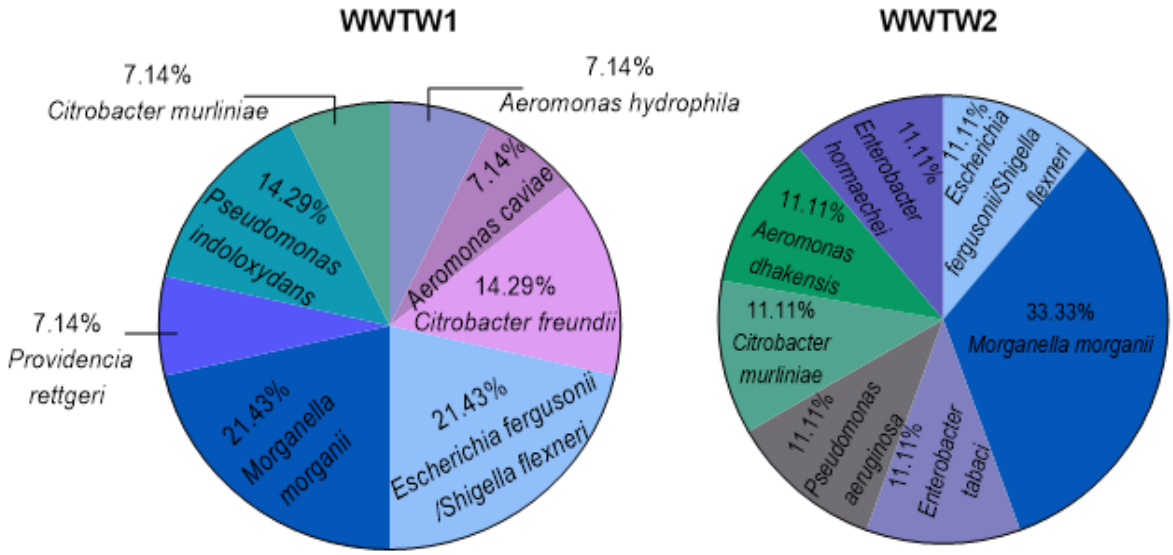

Figure 3.5. Species composition from different WWTW effluent samples previously exposed to antibiotics (WWTW1: n=12, WWTW2: n=7), and not previously exposed to antibiotics (WWTW1: n=2, WWTW2: n=2).

3.3.2. Sub-MIC impact on AMR

3.3.2.1. MIC determination for pure cultures and whole communities

Figure 3.6 shows that the concentrations of the selected antibiotics (AMX, SMX and CST) required to inhibit the growth of bacteria in WWTW samples were at least 2-fold lower in the effluent compared to influent and RAS of WWTW2. The MIC for CST and SMX was consistent throughout WWTW1 and between influent and RAS for WWTW2. The AMX MIC in the RAS of WWTW1 (16384 µg/ml) was double that of the rest of the WWTW samples (8192 µg/ml). The MIC for GM in WWTW2 effluent was 4-fold lower than that of the other WWTW2 matrices and all WWTW1 matrices.

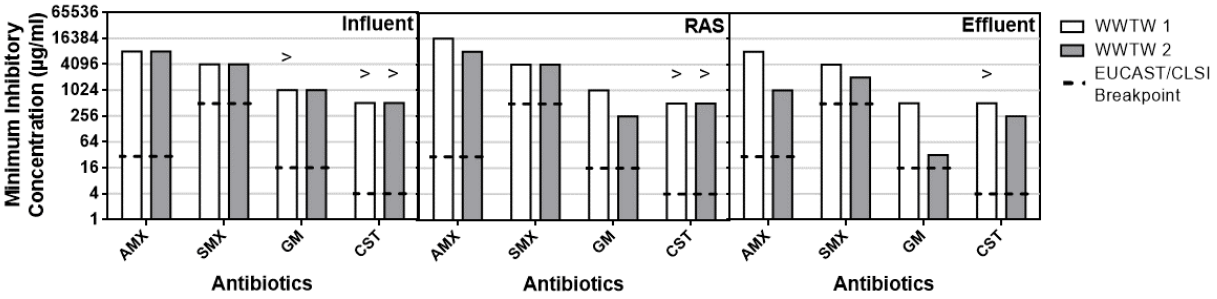

Figure 3.6. MICs of whole communities in influent, RAS and effluent samples from two WWTW for amoxicillin (AMX), sulfamethoxazole (SMX), gentamicin (GM) and colistin (CST). The greater than symbol (>) indicates growth still occurred in the highest concentrations used and as a result the MIC is greater than the value depicted by the bar.

In addition to the whole-community MICs depicted in Figure 3.6, Table A2.1 in Appendix A shows the individual MICs of AMX, SMX, GM and CST for the isolates randomly selected during the study. The MICs (Figure 3.7) for most isolates were higher than CLSI and EUCAST breakpoints for resistance (16384 µg/ml, 4096 µg/ml 2048 µg/ml for AMX, SMX and CST respectively); while the mode MIC for GM was on par with clinical breakpoints although most of the other isolates had MICs above this value (16 µg/ml) (Figure 3.7). Comparing the modes of isolate MICs (Figure 3.7) to whole community MICs (Figure 3.6), MICs of AMX and CST were higher for isolates than for whole communities, lower for isolates than whole communities for GM, and the same for single colonies and whole communities for SMX.

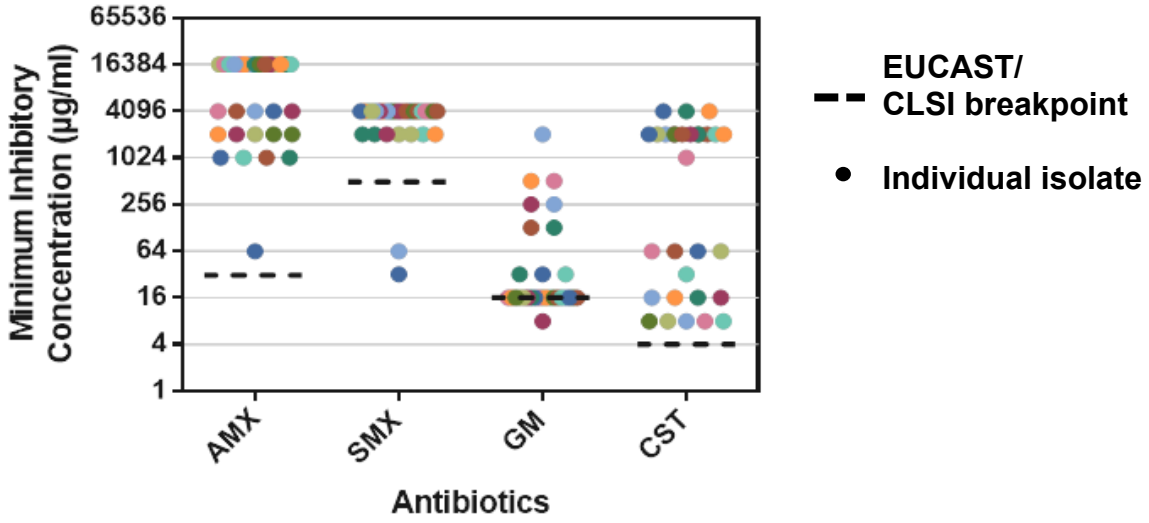

Figure 3.7. MICs of randomly selected isolates from WWTW, where each data point represents a single isolate. Mode MICs appear where data points cluster. Individual MICs, species identification and antibiotic exposure can be observed in Table S2.

3.3.2.2. qPCR measuring the effect of sub-MIC concentrations on ARG

Sul1, sul2 and bla_{KPC-1} were observed in all organisms isolated on antibiotic-free media (Figure 3.8). Except for two isolates showing exponentially higher (1×10^{13}) *sul2* copies, most isolates' gene copies clustered around the median. A paired t-test determined that changes in median gene copies after antibiotic exposure for all ARG were not significant ($p>0.05$). However, median gene copies were similar between *sul1* and *sul2* and increased after exposure to sub-MIC concentrations of sulfamethoxazole (average of 1.3×10^{8} copies before, increasing to 6.8×10^{8} copies after, while sub-MIC exposure to amoxicillin resulted in decreased median copy numbers of bla_{KPC-1} (3.9×10^{8} to 1×10^{8} gene copies/16S rDNA).

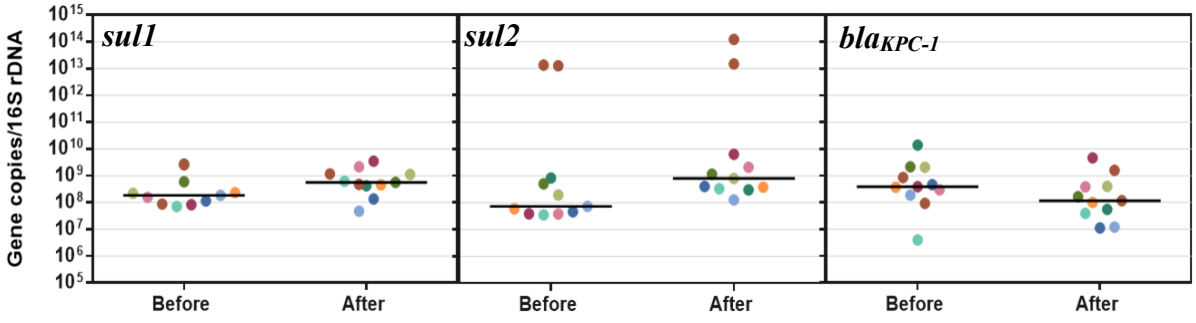

Figure 3.8. Copy number of antibiotic resistance genes/16S rDNA copies in colonies isolated on antibiotic free media before and after sub-MIC exposure to sulfamethoxazole (*sul1* and *sul2*) and amoxicillin (bla_{KPC}). The line for each data set represents the median.

3.4. Discussion

3.4.1. Changes in AMR during wastewater treatment

Multiple authors state that WWTW are hotspots for the dissemination of ARB and ARG (Berendonk et al., 2015, Guo et al., 2017, Dafale et al., 2020). This study shows that while these environments do indeed have waters that are contaminated with ARB and ARG, all tested genes, except *bla*$_{KPC-1}$ in WWTW2, were less prominent in the effluent than in the influent (Figure 3.1). Similarly, Figures 3.2 and 3.3 mostly show no net increase in ARB between the influent and effluent of the WWTW and while not statistically significant, there were notable decreases in ARB abundance. Similar results have been found in other studies (Duong et al., 2008; Lupan et al., 2017). This does not suggest that the ARB and ARG that are released into the environment from the WWTW effluent do not play a role in the transmission of AMR downstream, but demonstrates that the WWTW included in this study act to reduce the numbers of ARB and ARG entering the environment.

The antibiotic concentrations used for ARB selection in this study were selected based on CLSI resistance breakpoints (µg/ml range) and as a result, were much higher than what is observed in WWTW. Antibiotic levels in WWTW vary depending on the location, type, size of the facility, as well as operational efficiency. Antibiotic concentrations in local WWTW range from 1344.8 ng/L - 3612 ng/L for SMX in WWTW effluent (Archer et al., 2017; Suzuki et al., 2015); 1400 ng/L for AMX in influent (Watkinson et al., 2009); and 0.019-7.6 ng/L for GM (Löffler and Ternes, 2003; Tahrani et al., 2016), while to our knowledge no quantification of colistin in WWTW has been published. The concentration of a particular antibiotic that does not select for antibiotic resistance in bacteria is termed the predicted no-effect concentration (PNEC). The PNECs have been identified by Bengtsson-Palme and Larsson, (2016) with respective values for AMX, SMX, GM, and CST of 250 ng/L 16000 ng/L 1000 ng/L, 2000 ng/L. Therefore, it is reasonable to question whether concentrations of antibiotics in WWTW would need to be higher than these PNEC values to drive bacteria to develop resistance mechanisms and select for resistant communities – a point that requires debate and further analyses. Figure 3.2C-D shows that the positive controls (without antibiotics) also had increased abundance of bacteria after enrichment in antibiotic-free media, similar to trends observed for abundance of ARB enriched in antibiotic media. This could be expanded to a WWTW setting where the high labile nutrient source present is the driving factor for bacterial proliferation, conferring a r-selection evolutionary strategy. This survival strategy selects for organisms with higher growth rates and opportunists, which multiply rapidly and produce many

progeny that are less adapted for their particular environment (Vadstein et al., 2018). A suppressive pressure on whole-community growth rate (OD_{600}) for AMX, SMX and GM antibiotic enrichments in comparison to the MHB control was observed (Figure 3.2). In contrast, the OD_{600} for MAC in Figure 3.2C indicated that exposure to CST, resulted in a higher growth rate in influent and RAS microbial communities from WWTW1 compared to the control. This may indicate a highly adaptive community, with lower competitive pressure in these matrices. However, wavelength interference of the acidity indicator in MacConkey media may also play a role in the observed CST results. In addition, non-microbial components of wastewater may contribute to the turbidity of samples, as well as complex interactions in wastewater microbial metabolism influencing wavelength transmission in all media types. Thus, although there are also limitations with culture-based approach due to selection bias, culturing was selected as a quantification metric for subsequent analyses.

The percentage of ARG removed from influent to effluent was significantly higher in WWTW2 for *mcr3, sul1* and *sul2* (Figure 3.1). It was surprising that no $bla_{OXA\text{-}48}$ genes were identified as it has previously been identified in various countries, in multiple plasmid types and in South African environments (Bonomo, 2017; Ebomah and Okoh, 2020). In addition, a larger reduction in the abundance of resistant bacteria were observed in WWTW2 compared to WWTW1 (Figure 3.2 and 3.3) and the dominant species isolated from each WWTW also differed (Figure 3.5). Differences in capacity, sources of influent and type of effluent treatment strategies between the two WWTW may contribute to the result variation in ARB abundance and the dominant species observed between WWTW. WWTW1 uses a mixed flow approach for tertiary treatment, has a 200 ML/day capacity and was sampled upstream of the maturation pond. Due to the layout of this WWTW, a sample point downstream of the maturation pond was inaccessible. In contrast, WWTW2 uses a plug flow system for tertiary treatment, has a 38 ML/day capacity, and was sampled downstream of the maturation pond. WWTW1 is also 18 years older than WWTW2 and as a result, may have more lapses in effluent quality due to maintenance requirements. In a mixed flow system, contaminants such as antibiotics, ARB and ARG, are immediately distributed throughout the system, reducing the concentrations of these, and promoting persistence and proliferation, while in a plug flow there is less mixing, and contaminants in treated water are thus ‘pushed out’ of WWTW within its hydraulic retention time as new contaminants enter (Rumbaugh, 2014). Due to these treatment strategies, distributing ARB and ARG that persist after primary and secondary treatment in a mixed flow WWTW might promote persistence and proliferation, by homogenising with wastewater that

would otherwise not contain ARB or ARG. A plug flow system on the other hand, would allow ARB and ARG to pass through the tertiary treatment of the WWTW within its hydraulic retention time. Due to the fact that samples were collected after the maturation pond in WWTW2, aggregates of suspended solids may have settled in the maturation ponds, and may not be present in the collected sample, further reducing the ARB and ARG seen in the effluent of WWTW2 (Figures 3.1, 3.2, and 3.3). This study did not sample other parts of wastewater treatment such as solid waste prior to grit screening or waste sludge. Thus, ARB and ARG may still enter the environment including rivers, the ocean, and sediments through different avenues despite being seemingly reduced in wastewater effluent, as suggested by Sabri et al., 2020. Through this dissemination, other organisms could acquire these genes, increasing the spread of resistance (Czekalski et al., 2012). However, the amount of ARB in sludge has also been found to reduce significantly after anaerobic digestion due to the heat generated during the process that kills bacteria (Zhao and Liu, 2019).

The most prevalent species identified from a random sample of colonies, *M. morganii* (Figure 3.4), is intrinsically (innately) resistant to amoxicillin and colistin (Lui et al, 2016), both of which were used as a selective pressure and could explain why this organism was the most prominent in the samples. Several other genera (*Aeromonas, Escherichia, Citrobacter,* etc.) in Figure 3.4 are commensal bacteria in humans and/or animals and are frequently found in WWTW (Abbott, 2011; Bassetti et al., 2018; Janda and Abbott, 2010; Lee et al., 2009; Liu et al., 2016; Liu et al., 2017). The increased abundance of ARB in WWTW samples after high antibiotic exposure (Figures 3.2 and 3.3 C-D), and the selection of intrinsically resistant bacteria (Figure 3.4), may give insight into AMR emergence in an environmental setting. The emergence and dissemination of ARB and ARG within WWTW and surface waters has been proposed to be aggravated by chronic exposure of trace concentrations of non-metabolized antibiotics that are present (Kraemer et al., 2019). With sub-lethal exposure to antibiotics, most of the cells survive despite their growth being inhibited. As a result, to adapt to the exposure, small mutations such as single nucleotide polymorphisms may occur that have little phenotypic effect, however, upon continuous or frequent exposure, these mutations can accumulate resulting in emergence of resistance mechanisms (Andersson and Hughes, 2014). However, higher concentrations of antibiotics used in treating a patient or animal in a clinical or agricultural setting (or using them as supplements in animal feed) applies a stronger selection pressure within the microbiome, which has been shown to increase antibiotic resistance at a faster rate than lower antibiotic concentrations (Oz et al., 2014). As a result, selection of

intrinsically resistant pathogens and resistant microbial populations are more likely to occur in these microbial communities which may be excreted and enter WWTW in addition to surface waters. These organisms that persist in the WWTW effluent may pose health risks to humans and animals downstream of the WWTW. For example, *Aeromonas* spp. is a common fish pathogen and is frequently identified in wastewater, as well as on crops irrigated with treated wastewater (Figueras Salvat and Ashbolt, 2019). Farmworkers have been identified as high risk due to direct contact with contaminated water. Sprinkler systems used for irrigation also play a role in the spread of aerosolized pathogens (Adegoke et al., 2018). Several bacterial and viral outbreaks have occurred due to crops being contaminated after being irrigated with treated wastewater (Sano et al., 2016). These incidences reinforce the potential of antibiotic resistant organisms to disseminate into the environment and through the food chain if they are present in WWTW discharge.

3.4.2. Sub-MIC impact on AMR

The mode MICs for pure cultures for CST, AMX, SMX and GM (2048 µg/ml, 16384 µg/ml, 4096 µg/ml and 16 µg/ml respectively) (Figure 3.7) were 256, 2048, 8 times, and equal to the respective EUCAST MIC resistant breakpoint values, indicating that the microbes in these WWTW as a collective, were resistant to all the antibiotics tested in this study (Clinical and Laboratory Standards Institute, 2018; European Committee on Antimicrobial Susceptibility Testing, 2017). High MICs for bacteria isolated from wastewater have also been reported in numerous studies (Obayiuwana et al., 2018; Ovejero et al., 2017). Mode MICs for individual isolates (Figure 3.7) were found to be higher than the MICs for the whole community wastewater samples for AMX and CST (Figure 3.6). MICs for SMX were similar between whole community and pure cultures, and GM MICs were higher in whole communities. Increased prevalence of β-lactamase-producing bacteria has led to AMX having poor activity against Enterobacteriaceae and *Pseudomonas* spp. (European Committee on Antimicrobial Susceptibility Testing, 2010). As a result, AMX is combined with clavulanic acid, a β-lactamase inhibitor, for clinical treatment. Had clavulanic acid been combined with AMX in this study, lower MICs for pure cultures compared to whole communities may have resulted, as seen with the other antibiotics. However, referring to the previous example of resistance emergence occurring in the gut microbiome of animals and humans, individual members or a sub-group of these microbial communities that enter the environment through excretion, may develop higher MICs after being exposed to higher concentrations in the gut compared to organisms in the environment exposed to trace antibiotic concentrations, which can further be

diluted by rainfall and stormwater before entering WWTW due to these concentrations not exceeding PNEC values (Bengtsson-Palme and Larsson, 2016; Rolain, 2013). The lack of a significant change in resistant gene copy number in individual isolates randomly selected from WWTW after sub-inhibitory antibiotic exposure for 18-24 h is consistent with this picture (Figure 3.8). It has been shown previously that exposure of *E. coli* isolates to sub-MIC concentrations of various antibiotics result in increased MICs within 21 days of exposure through serial passage and that organisms exposed to higher concentrations (0.5 × MIC) had a higher occurrence of cross-resistance compared to isolates exposed to lower concentrations (1/8 × MIC) (Oz et al., 20. 14). As a result, whole communities that dominate regions of WWTW for biological treatment may have varying proportions of species intrinsically resistant to certain antibiotics. The abundance of these species may be lower than susceptible species and as a result, the MICs may appear to be lower than single isolates, obtained from antibiotic selective media, that have the necessary resistance mechanisms. This may explain the trends seen for the whole community and pure culture MICs in Figure 3.6 and 3.7. Thus, curbing AMR in the environment cannot be addressed by confronting WWTW alone and needs to be stewarded carefully from the point of usage. The wide range of antibiotic reactivities, resistance genes and mechanisms that would respond differently to various treatments lead to a range of AMR issues. Potential outbreaks in humans or animals with organisms identified in this study would be untreatable with the target antibiotics due to the MIC values seen in Figure 3.7.

While problem areas within the treatment process in terms of AMR should be identified, and alternative technologies implemented that promote more effective removal of all harmful contaminants, it is worth reminding that WWTW are the receiving bodies rather than the original source of antibiotics and ARB that carry ARG (Kümmerer, 2009). In addition to the entry of ARB, chemicals that are used in industry may contribute AMR. Chlorine treatment has been found to increase the permeability of bacterial cells, allowing increased horizontal gene transfer (HGT) of ARG between donor and recipient cells (Guo et al., 2015). Entry of chlorine and other disinfectants used for cleaning hospitals, abattoirs, swimming pools etc. into WWTW may increase HGT in WWTW with a dense microbial load, such as activated sludge, or at the original source of contamination.

3.5. Conclusion

It is evident that ARB are abundant in WWTW regardless of the stage of the treatment process. Effluent ARB species detected in this study can be disseminated via downstream surface waters in the environment, which can be used for irrigation, livestock watering, and other repurposing. However, while ARB and ARG were present in chlorinated effluent, the numbers were reduced compared to the influent, suggesting that WWTW assist in reducing dissemination of AMR in the environment, and it appears plausible that the sub-MIC exposure in WWTW had little effect on the emergence of ARB and ARG. As a result, a more proactive solution in tackling AMR would be to mitigate the entry of these ARB and ARG into the WWTW altogether and to encourage the development of antibiotics that are metabolised more readily to prevent excretion of the parent compound and active metabolites into WWTW. In addition, providing access to basic hygiene and sanitation amenities in developing countries, promoting food-animal health, and implementing potential incentives or consequences to encourage adherence to disposal legislation and to reduce the use of antibiotics, where possible may assist in mitigating the entry of ARB and ARG into water sources. Education on AMR and daily routines that play a role in the functioning of the WWTW could aid in the removal efficiency of antimicrobials and other contaminants and promote a proactive mindset in addressing the issue of AMR in the environment.

3.6. Acknowledgements

Thank you to Edward Archer, Mercia Volschenk, and the City of Cape Town for assisting with and permitting sampling of WWTW. Thank you to the Central Analytical Facility at Stellenbosch University for DNA sequencing. This work is based on the research supported in part by the National Research Foundation of South Africa (Grant numbers: 118159 and 130527) and the Water Research Commission (Project No. K5/2733).

APPENDIX A

SUPPLEMENTARY MATERIAL FOR CHAPTER 3

Wastewater treatment works: A last line of defence for preventing antibiotic resistance entry into the environment

A.1. Supplementary Methods

Table A1.1: Target antibiotic resistance gene sequences and control gene ratio

Gene sequence (5'-3')	Product size	Proportion of total plasmid ($(bp_t)/ bp_p$)	NCBI Accession number
***bla*$_{OXA-48}$ gene** AATAGCTTGATCGCCCTCGATTTGGGCGTGGTTAAGGATGA ACACCAAGTCTTTAAGTGGGATGGACAGACGCGCGATATC GCCACTTGGAATCGCGATCATAATCTAATCACCGCGATGAAA ATATTCAGTTGTGCCTGTTTATCAAGAATTTGCCCGCCAAAT TGGCGAGGCACGTATGAGCAAGATGCTACATGCTTTCGATT ATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGG CTCGACGGTGGTATTCGAATTTCGGCCACGGAGCAAATCAG CTTTTTAAGAAAGCTGTATCACAATAAGTTACACGTATCGG AGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGA AGCCAATGGTGACTATATTATTCGGGCTAAAACTGGATACT CGACTAGAATCGAACCTAAGATTGGCTGGTGGGTCGGTTGG GTTGAACTTGATGATAATGTGTGGTTTTTTGCGATGAA (488bp)	438	0.292	AY236073
***bla*$_{KPC-1}$ gene** CCCGGCCGGGCTGACGGCCTTCATGCGCTCTATCGGCGATA CCACGTTCCGTCTGGACCGCTGGGAGCTGGAGCTGAACTCC GCCATCCCAAGCGATGCGCGCGATACCTCATCGCCGCGCGC CGTGACGGAAAGCTTACAAAAACTGACACTGGGCTCTGCA CTGGCTGCGCCGCAGCGGCAGCAGT (188bp)	138	0.092	AF297554
***Mcr-3* gene** GTGTCAGTGGGGCGCAACAATTCAAACCTCCAGCGTGAGAT TGTTCCAGCCAATTTCGTTAATAGTACCGTTAAATACGTTTA CAATCGTTATCTTGCTGAACCAATCCCATTTACAACTTTAGG TGATGATGCAAAACGGGATACTAATCAAAGTAAGCCCACG TTGATGTTTCTGGTCGTTGGTGAAACCGCTCGTGGTAAAAA TTTCTCGATGAAT (219bp)	169	0.113	MH077952.1
***Sul1* gene** TGCGGACGTAGTCAGCGCCATTGCCGATCGCGTGAAGTTCC GCCGCAAGGCTCGCTGGACCCAGATCCTTTACAGGAAGGCC AACGGTGGCGCCCAAGAAGGATTTC (107bp)	67	0.045	CP031449.2
***Sul2* gene** CCCGGACCACGGCCTGTGAGCGCGCGCAGAAAGGATTTGC GCGAAACAGACAGAAGCACCGGCAAATCGAAGCGCAGCCG CAATTCATCGAACCGCGCCA (100bp)	60	0.04	MK165650.1

A.1.1 Generation of standard curves and calculating gene copies

Rather than conducting a PCR on the control plasmid to amplify each target gene for each qPCR run, the constructed control plasmid for target resistance genes was serially diluted (1:10) in TE buffer and a standard curve was generated in triplicate for each target gene. The

proportion of base pairs that each target gene contributed to the whole plasmid was determined by dividing the number of base pairs in the target gene (bp_t) by the total number of base pairs in the plasmid (bp_p) (Table A1.1). The copy number of the target genes in the complete plasmid in the control qPCR reactions was calculated using Equation A1 and multiplied by each respective gene ratio.

Where *ng* is the mass of DNA of the target gene added to the PCR reaction in nanograms,

$$\textbf{Number of copies} = \frac{\textbf{ng} \times \textbf{Avogadro's constant}}{\textbf{bp} \times \textbf{conversion factor} \times \textbf{Average mass of bp}} \quad \text{(A1)}$$

Avogadro's constant is valued at 6.022×10^{23}, *bp* is the size of the target gene in base pairs, and *conversion factor* and *average mass of bp* are both constants valued at 1×10^{9} and 660 Da, respectively.

The copy numbers of the control genes were multiplied by the gene ratio determined previously (Table A1.1). The result was then logged and plotted against the Ct values to generate a standard curve (Figure A1.1) and linear equation (Table A1.2). The average of the resulting Ct values of each target gene for samples were substituted into the linear equation of each gene determined from the standard curve. In order to obtain the copy number of each gene, the inverse log was calculated, and this value was then subsequently divided by the copy number of 16S rDNA genes that was detected for normalisation.

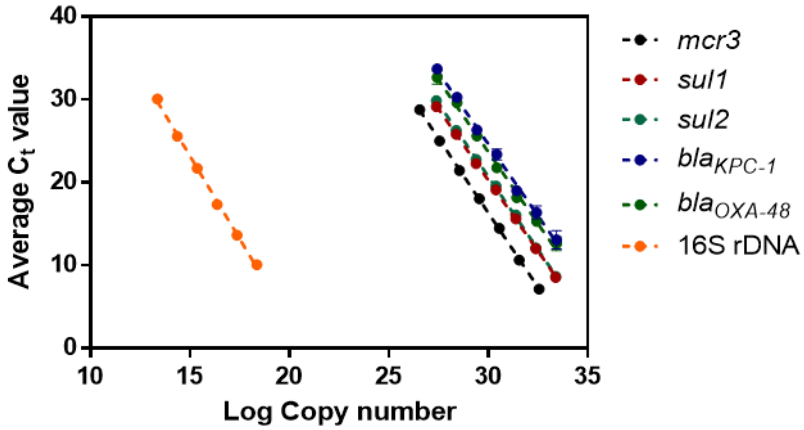

Figure A1. 1. Standard curves for target antibiotic resistance genes used in whole-community and single colony analysis.

After qPCR amplification of the positive control genes and generation of the standard curve, a straight-line equation was determined, and the efficiency of the PCR reaction was determined by the gradient of the curve using Equation (A2).

$$\textbf{qPCR efficiency} = (10^{-1/\text{slope}} - 1) \times 100 \quad \text{(A2)}$$

Log reduction of gene copies from influent to effluent were then calculated using Equation A3.

$$R = \log_{10}\left(\frac{c_a}{c_b}\right) \quad \text{(A3)}$$

Where R is the log reduction, c_a is the number of gene copies in the influent sample and c_b is the number of gene copies in the effluent sample.

Table A1. 2. Standard curve equation for gene copy determination

Gene	Equation (y=)	R^2	Efficiency (%)
Mcr3	-3.593x+124.1	0.9996	90
Sul1	-3.430x+123.2	0.9996	96
Sul2	-3.522x+126.4	0.9991	92
Bla_{KPC-1}	-3.462x+128.5	0.9912	94
Bla_{OXA-1}	-3.466x+127.7	0.9935	94
16S rDNA	-4.003x+83.27	0.9983	78

Table A1. 3. DNA concentration from wastewater samples

WWTW	Sample site	DNA concentration (ng/µl)
1	Influent	4.7
	RAS	3.1
	Effluent	4.4
2	Influent	3.4
	RAS	5
	Effluent	3.5

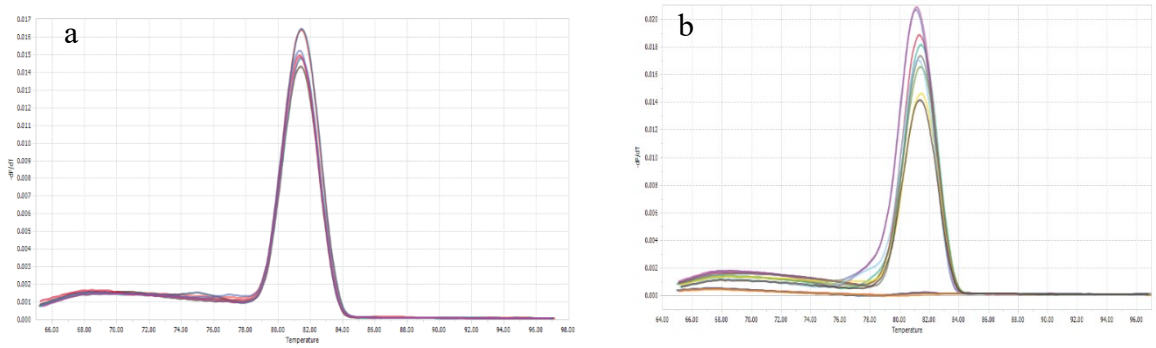

Figure A1. 2. Melt curves of *mcr3* gene for a) control gene in plasmid and b) WWTW samples.

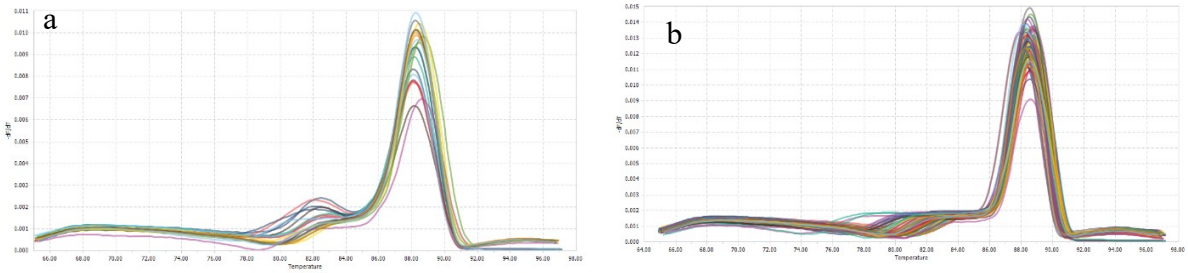

Figure A1. 3. Melting peaks of 16S rDNA in a) WWTW samples and b) single colony samples.

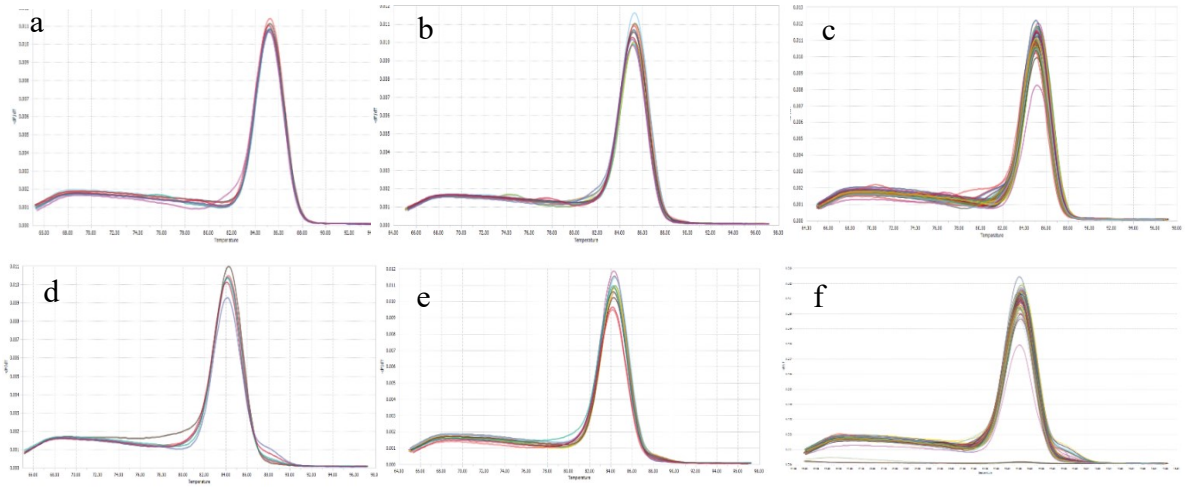

Figure A1. 4. Melting peaks of a) *sul1* control, b) *sul1* WWTW samples, c) *sul1* single colony samples, d) *sul2* control, e) *sul2* WWTW, f) *sul2* single colonies.

A.1.2 DNA extraction protocol adapted from (Crouse and Amorese, 1987)

1. Harvest 2 ml of an overnight bacterial culture by centrifugation at 2470 x g for 3 minutes in 2 ml microfuge tubes. Wash once with sterile TE buffer (*be gentle and quick - some bacteria may tend to start lysing in TE*).

2. Resuspend cell pellets in 500 µl SET buffer (25% sucrose, 2mM EDTA, 50mM Tris pH8).

3. Add protease K to 1 mg/ml. Incubate at 37 °C for 30 minutes.

4. Split the cell suspension into 2 x 500 µl in two microfuge tubes and add 500 µl TE to each.

6. Lyse cells with 50 µl 10% SDS. *Mix gently- the solution should become clear and gloopy*.

7. Incubate at 50 °C overnight. *This is step allows the proteinase K to degrade protein debris*.

8. Add 500 µl 7.5 M ammonium acetate to each tube and mix well. Hold at room temperature for 60 minutes. *This step is used to precipitate protein from solution*.

9. Spin at 16 700 x g for 15 minutes at room temperature to precipitate proteins. Place 500 µl of the supernatant into clean 2 ml microfuge tubes and add 2 volumes of 100% ethanol.

10. Leave overnight at 4 °C.

11. Centrifuge at 16 700 x g for 30 minutes at room temperature.

12. Carefully remove the supernatant from the DNA pellet and wash the pellet with 70% ethanol. Centrifuge at 16 700 x g for 5 minutes. Remove the supernatant and allow pellet to dry completely.

13. Resuspend the DNA of one tube in 200 µl TE buffer and combine with the other sample tube. Add RNase (final concentration 100 ng/ml). Incubate at 37 °C for 30 minutes.

A.2. Supplementary Results

Concentrations 2-fold lower than the MIC for each antibiotic were selected as the concentration to use for isolation of antibiotic resistant bacteria for subsequent experiments.

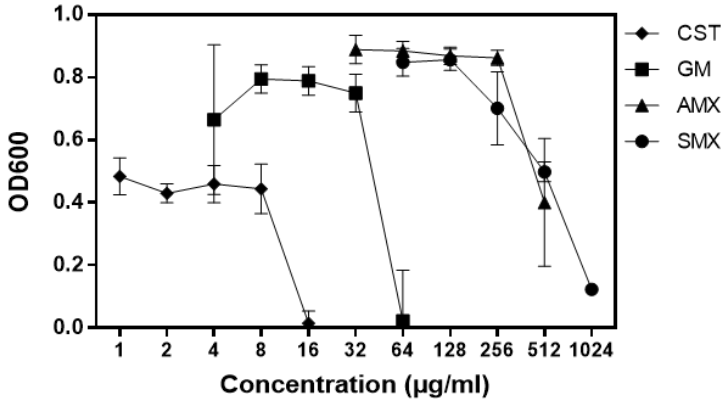

Figure A2.1. Optical density measured at 600nm of a RAS culture exposed to antibiotic concentration ranges to determine minimum inhibitory concentration to mixed RAS cultures.

In most samples, gene copies per 16S rDNA can be ranked in the following order: *sul1* > *sul2* > bla_{KPC-1} > *mcr3* > bla_{OXA-48}.

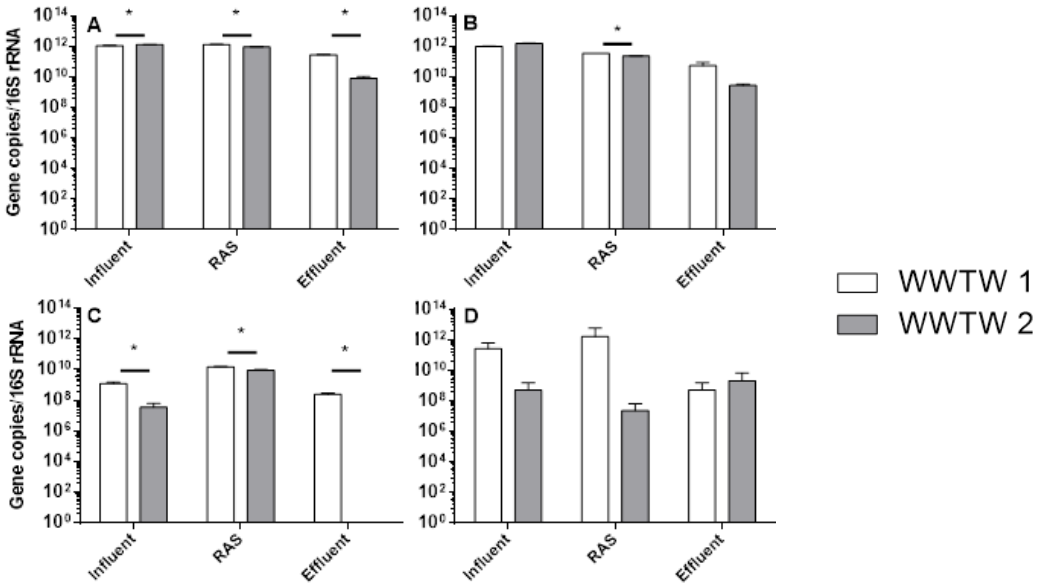

Figure A2. 2. Copy number of plasmid mediated resistance genes per 16s rDNA copy number for sulfamethoxazole, (A) *sul1* and (B) *sul2*, colistin resistance gene (C) *mcr3*, and β-lactam resistance gene (D) bla_{KPC-1} in influent, return activated sludge (RAS) and effluent samples obtained from two WWTW. A multiple t-test was used to calculate significant differences between WWTW shown by the line and asterisk (*).

Table A2. 1. Metadata for isolated single colonies including identities, MICs to colistin (CST), gentamicin (GM), amoxicillin (AMX) and sulfamethoxazole (SMX), and concentration of extracted DNA for qPCR

						MIC (µg/ml)				DNA concentration (ng/µl)		
Isolate number	**Species**	**WWTW**	**Season**	**Sample**	**Antibiotic isolated on**	**CST**	**GM**	**AMX**	**SMX**	**Before Sub-MIC exposure**	**After sub-MIC AMX exposure**	**After sub-MIC SMX exposure**
1	*Morganella morganii*	2	Summer	Effluent	CST	>2048	128	1024	4096	63.0		
2	*Pseudomonas aeruginosa*	2	Summer	Effluent	AMX	2048	8	4096	2048	240.5		
3	*Morganella morganii*	2	Summer	Effluent	AMX	>2048	16	2048	4096	44.8		
4	*Aeromonas caviae*	1	Summer	Effluent	SMX	2048	32	1024	2048	17.7		
5	*Aeromonas hydrophila*	1	Summer	Effluent	SMX	2048	16	64	32	24.7		
6	*Citrobacter freundii*	1	Summer	Effluent	AMX	2048	16	16384	2048	10.4		
7	*Escherichia fergusonii/Shigella flexneri*	1	Summer	Effluent	AMX	64	16	4096	4096	358.6		
8	*Morganella morganii*	1	Winter	Effluent	CST	2048	16	2048	4096	21.7		
9	*Morganella morganii*	1	Winter	Effluent	CST	>2048	32	16384	4096	131.9		
10	*Citrobacter freundii*	1	Summer	Effluent	GM	2048	256	16384	4096	269.9		
11	*Morganella morganii*	1	Summer	Effluent	CST	>2048	16	16384	4096	22.3		
12	*Escherichia fergusonii/Shigella flexneri*	2	Summer	Effluent	GM	2048	256	16384	4096	79.9		
13	*Citrobacter murliniae*	2	Summer	Effluent	SMX	2048	16	16384	4096	42.4		
14	*Aeromonas dhakensis/caviae/enteropelogenes*	2	Summer	Effluent	SMX	4096	128	16384	2048	358.8		
15	*Providencia rettgeri*	1	Winter	Effluent	SMX	4096	32	4096	4096	6.2		
16	*Morganella morganii*	2	Summer	Effluent	GM	>4096	512	16384	4096	1140.4		
17	*Escherichia fergusonii/Shigella flexneri*	1	Summer	Effluent	GM	<8	512	16384	4096	73.4		
18	*Pseudomonas indoloxydans*	1	Winter	Effluent	AMX	<8	16	16384	2048	198.4		
19	*Pseudomonas indoloxydans*	1	Winter	Effluent	AMX	<8	16	16384	2048	89.5		
20	*Shigela sonnei/flexneri*	1	Winter	Influent	None	16	16	4096	4096	224.7	96.5	54.9
21	*Klebsiella pneumoniae*	2	Winter	Influent	None	64	16	4096	4096	344.7	57.5	63.2
22	*Klebsiella quasipneumoniae*	2	Winter	Influent	None	16	16	2048	4096	184	64.2	61.9
23	*Escherichia fergusonii/Shigella flexneri*	1	Winter	Effluent	None	<8	16	2048	4096	566.2	101.0	68.3
24	*Citrobacter freundii/murliniae*	1	Winter	Effluent	None	16	16	16384	4096	174.3	65.7	71.1
25	*Enterobacter tabaci*	2	Winter	Effluent	None	64	16	1024	4096	48	57.4	67.5
26	*Klebsiella quasipneumoniae*	2	Winter	RAS	None	16	16	2048	4096	60.1	66.9	115.3
27	*Klebsiella pneumoniae*	2	Winter	RAS	None	1024	16	16384	4096	71.4	108.9	42.7
28	*Enterobacter hormaechei*	2	Winter	Effluent	None	64	16	16384	2048	81.6	448.1	84.6
29	*Enterobacter asburiae*	1	Winter	RAS	None	32	16	1024	4096	122.9	66.0	99.7
30	*Enterobacter tabaci*	1	Winter	Influent	None	<8	>**2048**	16384	64	186.6	71.0	97.5
					Mode	2048	16	16384	4096			
					Max	4096	512	16384	4096			
					Min	8	8	64	32			

CHAPTER 4

EXPLORING ENVIRONMENTAL ANTIBIOTIC RESISTANCE IN AN URBAN HYDROLOGICAL CYCLE

Abstract

Inadequate amenities and the inability for basic infrastructure to keep up with the rapid rate of urbanisation in developing countries have led to deterioration of environmental water quality. With the increasing emergence of antimicrobial resistant bacteria (ARB), and the risk it poses to public health, there is growing concern that water pollution due to insufficient sanitation may become a major contributor to the spread of antimicrobial resistance (AMR). Here, baseline data on how different anthropogenic factors are impacting the level of antibiotic resistant strains and genes in the urban hydrological cycle in Stellenbosch, South Africa is provided. Gram-negative bacteria were recovered from aqueous, biofilm and sediment samples representing informal settlements, residential, industrial, and agricultural settings, as well as a municipal wastewater treatment plant, over a period of a year by selectively culturing on media containing carbapenems, colistin, gentamicin and sulfamethoxazole. A metagenomic approach was adopted to determine community profiles and dominant AMR genes at various sites while carbapenem resistant colonies were selected and characterised using whole genome sequencing (WGS). The prevalence of resistance to carbapenems and colistin was similar across all sampling sites, and the percentage of bacteria resistant to these antibiotics increased at agricultural sites. On the other hand, a higher percentage of bacteria resistant to gentamicin and sulfamethoxazole were isolated at sites impacted by domestic runoff. Multidrug efflux pumps were the most prominent resistance genes across all sites while among the dominant target resistance genes detected were *cph* and *imiH* (carbapenems), *bacA* (colistin), *aph 3''Ib, Aph 6-Id* and *aadA* (gentamicin), and *sul1* (sulfamethoxazole).

4.1. Introduction

With the rise of AMR, predicted to cause 10 million annual deaths by 2050 (O'Neill, 2014), together with the One Health approach (World Health Organisation, 2017a), the role of environmental waters as a reservoir of AMR has gained increased recognition. Most studies investigating AMR in the urban water cycle focus on wastewater treatment works (WWTW), and many have deemed these sites as hotspots for the emergence of ARB and the source of AMR in the environment (Pazda et al., 2019; Rizzo et al., 2013). However, an overlooked area of study for ARB emergence and transmission lies outside of water treatment facilities and rather in the source, or usage parts of the urban water cycle (Figure 4.1). In low- and middle-income countries (LMIC) such as South Africa (SA), WWTW are often poorly maintained and receive influent at, or above operating capacity, and therefore do not produce an effluent that is compliant to wastewater treatment regulations resulting in high levels of contamination in receiving waters (Herbig, 2019). Furthermore, inadequate sanitation and related infrastructure in rapidly expanding informal settlements, often combined with poor maintenance of systems required for the delivery and collection of water, result in bypassing the treatment process. As a result, usage impacts the health of the source and vice versa, as indicated by the orange arrows in Figure 4.1. Bucket toilets and open sewers are amongst the many unsanitary practices that occur in the informal settlements and contribute greatly to environmental pollution (Harvey, 2018).

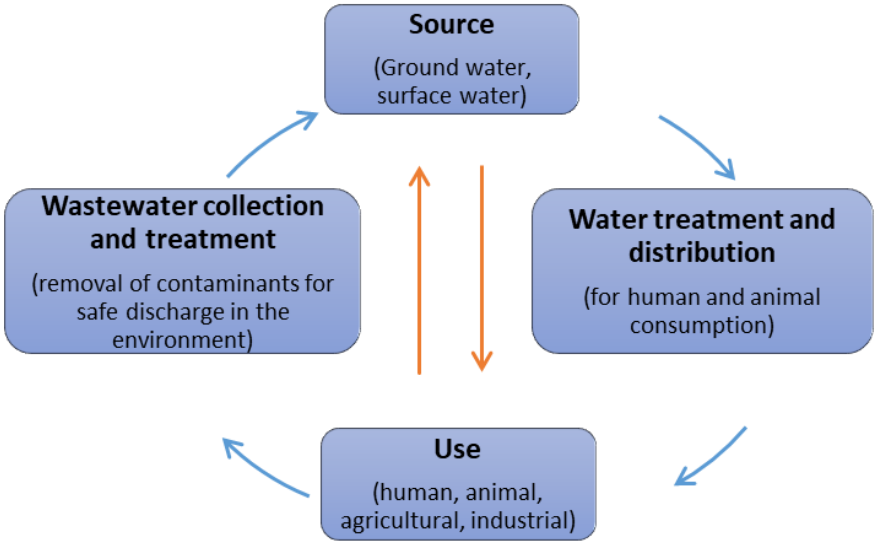

Figure 4.1. Summary of the urban water cycle. The conventional urban water cycle is depicted with blue arrows. Orange arrows indicate pathways that may occur in low middle-income countries due to lack of amenities. Multiple sources of water are used; lakes, dams and rivers contribute to surface water sources, while well points and boreholes give access to ground water. Adapted from Sowby, (2014).

Increased use of antibiotics and development of resistance has led to last resort antibiotics such carbapenems and colistin being used more frequently, and as a result, resistance to these antibiotics has become a concern due to the limited treatment options available (Codjoe and Donkor, 2017; Labuschagne et al., 2016; McKenna, 2013). In SA, 80% of available antibiotics are used for agricultural, livestock, and domestic animal purposes (National Department of Health, 2016), not only to treat infection, but also for preventative measures, and to facilitate growth promotion (Laxminarayan et al., 2013). As antibiotics for livestock are mostly added to animal feed, they have a direct effect on the animal's gut microbiota. Therefore, the animals might act as a reservoir for resistant bacterial populations that can be excreted into the environment or have a zoonotic potential (Dafale et al., 2020; You and Silbergeld, 2014). Focusing on AMR in a defined section of the urban hydrological cycle such as WWTW, may lead to a biased opinion that singles out WWTW as the primary source of AMR; however, WWTW receive their influent from various sectors of society that could ultimately be the source of ARB and antibiotic resistance genes (ARG). This study aimed to consider the various sectors of an urban setting that may contribute to AMR, and which potentially serve as hotspots by determining resistance profiles from sites that are influenced by agriculture, industry, hospitals, as well as formal and informal residential dwellings.

4.2. Materials and Methods

4.2.1. Sample sites and collection

The Stellenbosch region near Cape Town, SA was selected as the site for this study due to the various societal sectors in close proximity. Informal settlements, winelands, and agriculture, as well as formal residential and industrial sectors are located within a 7 km radius of the town centre. Aqueous, sediment and biofilm samples were collected from 10 sampling sites representing these diverse pressures every other month for a period of one year (July 2018 - May 2019) (Figure 4.2).

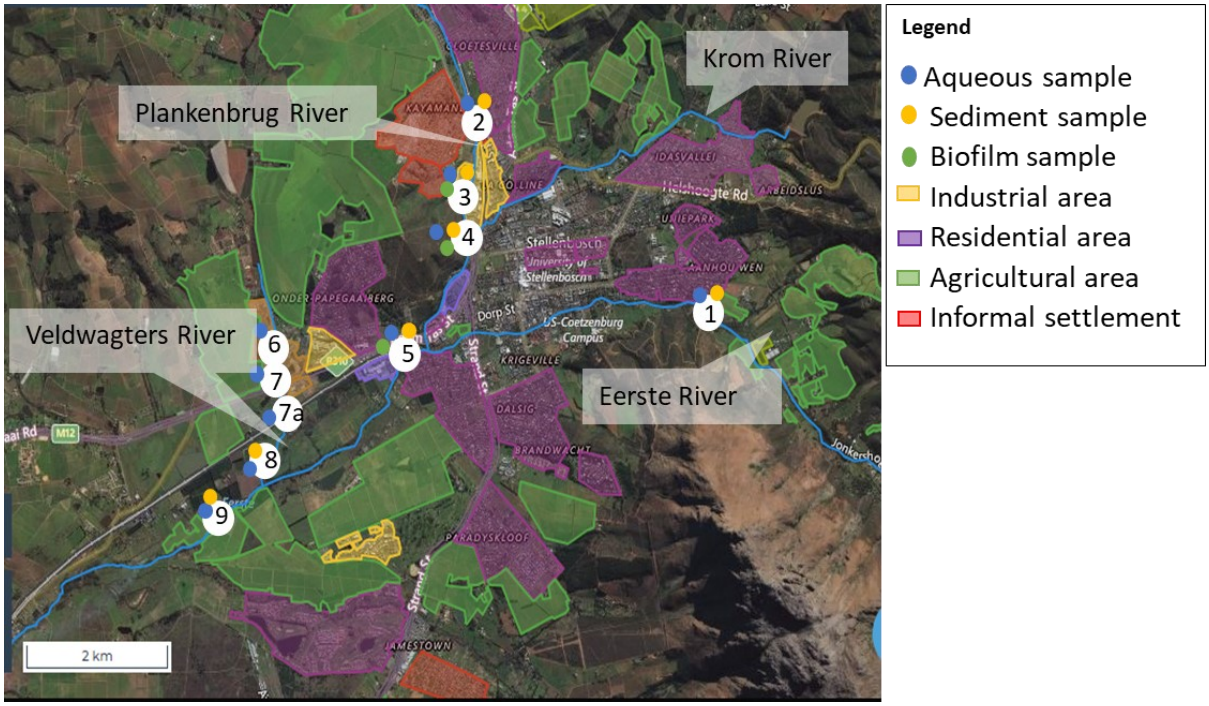

Figure 4.2. Map showing Stellenbosch rivers and sample sites. 1- Eerste River, before influence of most sectors and used as a pristine site, 2- Plankenbrug river, upstream of informal settlement, 3- Plankenbrug river, downstream of informal settlement, 4- Plankenbrug river, industrial influence, 5- convergence of Eerste and Plankenbrug rivers, 6- wastewater treatment plant influent, 7- wastewater treatment plant effluent, 7a-point of treated effluent discharge, 8- wastewater effluent enters into Veldwagters river, 9- convergence of Eerste and Veldwagters rivers. For reporting purposes, sites 2, 3 and 4 are combined and referred to as "industrial and informal settlement" and sites 7 and 7a are combined as "wastewater effluent".

Two aqueous samples (blue dots in Figure 4.2) were collected in sterile Schott bottles (one in 250 ml; all sampling sites, and one in 1L; site 1, 2, 3, 6, 8) from the mid-point between the surface and the bottom of the river. Biofilm samples were scraped from the under-side of rocks in the river using a toothbrush rinsed with 70% ethanol. The collected samples were then placed in sterile 50 ml falcon tubes containing 15 ml phosphate buffered saline (PBS). Collection of sufficient biofilm material was possible at sites 3, 4 and 5 indicated by green dots in Figure 4.2. Sediment samples (yellow dots in Figure 4.2) were collected from all sites, except the WWTW (sites 6, 7 and 7a). Sediment samples were collected from the bottom of the riverbed or at the lowest depth possible in 15 ml falcon tubes. All samples were transported on ice back to the laboratory where they were stored at 4°C until processing. All processing occurred within 6 hours of returning to the lab.

4.2.2. Enrichment of highly resistant Gram-negative bacteria

Gram-negative bacteria were targeted for ARB enumeration in this study. Four antibiotics considered a priority for study by the World Health Organisation were selected; two are last resort antibiotics (carbapenems and colistin) and two are frequently used in a South African setting (gentamicin and sulfamethoxazole).

Serial dilutions (1:10) from each 250 ml aqueous sample were made in PBS, and 100 µl of each dilution was spot plated in duplicate onto MacConkey agar (which served as positive control selecting for culturable Gram-negative bacteria present in the sample), SuperCARBA agar (CHROMagar) to select for carbapenem resistant Gram negative bacteria, and MacConkey agar supplemented with 512 µg/ml sulfamethoxazole (GLS), 32 µg/ml gentamicin sulfate (Melford) and 8 µg/ml colistin sulfate (Sigma-Aldrich). Antibiotic concentrations used were higher than the CLSI and EUCAST resistance breakpoints (Clinical and Laboratory Standards Institute, 2018; European Committee on Antimicrobial Susceptibility Testing, 2017) to select for highly resistant organisms in the sample.

Each sediment and biofilm sample was distributed into 2 ml microfuge tubes and centrifuged at 9900 × g for 10 min. The supernatant was discarded, and 100 mg wet weight was added to 900 µl PBS to make a 1:10 dilution. Each sample was vortexed thoroughly, a dilution series was made, and spot plates were performed as above.

All plates were incubated at 37 °C for 18-24 h and resulting colonies were enumerated. CFU/ml for aqueous, and CFU/g for sediment and biofilm were calculated. The percentage of culturable Gram-negative bacteria resistant to each antibiotic was calculated as follows:

$$\% \text{ resistance} = \frac{\text{CFU/ml(g) on antibiotic agar}}{\text{CFU/ml(g) on MacConkey agar}} \times 100$$

4.2.3. Whole genome sequencing

Single colonies from each site and matrix were picked from SuperCARBA agar plates previously enumerated. Species were presumed chromogenically from manufacturers recommendations: *E. coli* (pink), *P. aeruginosa* (cream to green), *A. baumannii* (cream), *Klebsiella* spp. (blue). Each selected colony was re-streaked on SuperCARBA media until a pure culture was obtained and was subsequently inoculated into 5 ml Terrific Broth and incubated overnight at 37 °C. Genomic DNA from each overnight culture was extracted using a method adapted from Crouse and Amorese, 1987. Complete protocol can be found in Appendix A.1.2

Extracted DNA from 132 isolates was sent to MicrobesNG (Birmingham, UK) where Illumina MiSeq sequencing (2×250 bp paired end reads) was conducted with in-house quality control (adapter trimming with Trimmomatic v0.30, with a sliding window quality score cut-off value of Q15). Taxonomical profiling was performed with Kraken2 (Wood et al., 2019) and MetaPhlAn2 (Segata et al., 2012). ARG were identified using the Comprehensive Antibiotic Resistance Database (CARD) and ABRicate (v1.0.1).

4.2.4. Whole community analysis

The 1L aqueous samples were filtered through 0.22 µm mixed cellulose ester filters (MilliPore). The turbidity of the samples determined the volume filtered (Appendix B, Table B1.3). The filtrate was discarded, and the filter was placed in a petri dish containing 4 ml citrate buffer (0.3M, pH3.5) for 3 min (Waso et al., 2016). Remaining solid matter was scraped off the filter using a sterile blade, and the filter was discarded. The solution was placed in microfuge tubes and centrifuged at 15000 ×g for 2 min. The pellet was washed with PBS and spun again, after which it was resuspended in 200 µl PBS. Total DNA was extracted from prepared aqueous samples, as well as 200 mg (wet weight) of the biofilm and sediment samples prepared as in 4.2.2 using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (ZymoResearch).

Extracted DNA from 16 metagenomic samples was sent to MicrobesNG (Birmingham, UK) and sequenced as described for the single colonies. Taxonomical profiling was performed with Kraken2, together with the Bracken procedure (Lu et al., 2017) with the Genome taxonomy database for improved performance (GTDB) (Méric et al., 2019). ARG were identified using the Comprehensive Antibiotic Resistance Database (CARD) and Resistance Gene Identifier (RGI; 5.1.1).

For additional information on the genomic and metagenomic samples see Appendix B, Table B1.1 and Table B1.3, respectively.

4.2.5. Statistical Analysis

Repeated measures ANOVA with the assumption of compound symmetry over months was used. Fisher's Least Significant Difference (LSD) post hoc test was performed to detect significant differences between sites over time using Statistica Software (TIBCO Software Inc. (2019)). Data Science Workbench (v13) was used to compare dates, sites, matrices, and antibiotics. A p value threshold of 0.05 was used for significance.

4.3. Results

4.3.1. Percentage resistance

Six samples per site were taken for this study over the course of one year. The greatest monthly variation in phenotypic antibiotic resistance was evident at the pristine site (site 1) in January 2019 and in wastewater effluent (site 7 / 7a) in September 2018, March, and May 2019. No resistant bacteria were observed in the wastewater effluent in July and November 2018 and January 2019. There was a general decrease in the percentage of resistant bacteria in the warmer months (November 2018 and January 2019; Figure 4.3).

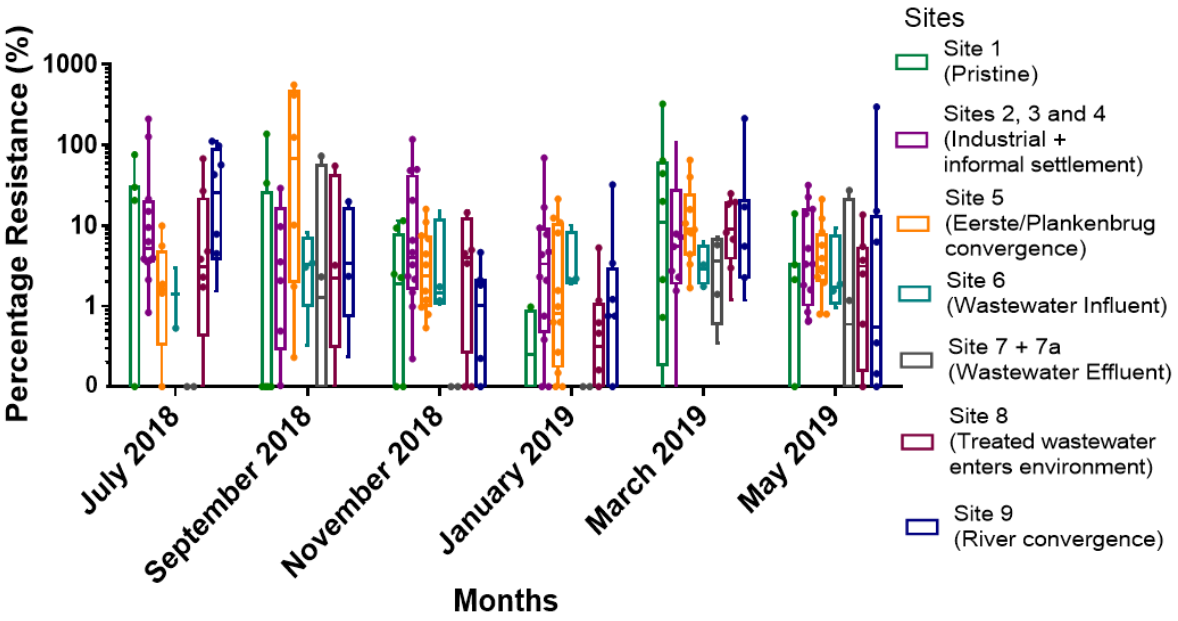

Figure 4.3. Average percentage of antibiotic resistant bacteria (all antibiotics and matrices) at each site for each sampling event.

The median percentage resistance for all matrices and sample sites ranged from 1.0 – 40% for carbapenems, 0.5 – 20% for colistin, 0.1 – 3% for gentamicin and 0.5 - 40% for sulfamethoxazole (Figure 4.4). The percentage of bacteria resistant to gentamicin across all sites were significantly lower than those resistant to carbapenems ($p < 0.05$) and colistin ($p < 0.01$), while a significantly higher percentage of colistin resistant bacteria was observed compared to sulfamethoxazole resistant bacteria ($p < 0.05$). Carbapenems and colistin had a similar range of median percentages of resistant bacteria across all sample sites, barring for wastewater effluent where colistin had a lower range compared to carbapenems. The percentage of resistant bacteria in wastewater effluent samples for all antibiotics varied greatly over sampling events compared to that of wastewater influent (Figure 4.4). A lower percentage of bacteria were resistant to gentamicin and sulfamethoxazole in aqueous matrices at the

pristine site (site 1), entry of treated wastewater into environment (site 8) and the river convergence (site 9) compared to the industrial and informal settlement sites (sites 2, 3, 4), Eerste/Plankenbrug convergence (site 5) and wastewater influent (site 6). There were no culturable bacteria resistant to sulfamethoxazole at the pristine site.

When taking all data, regardless of matrix, timepoint or antibiotic into consideration, site 9 had the highest percentage of ARB. Monthly variations did occur however, and each antibiotic had a different distribution (Figure 4.4). Overall, there was no significant difference in the percentage of culturable Gram-negative bacteria between the aqueous, sediment and biofilm samples ($p > 0.05$). In general, sediment samples had the highest median percentage of antibiotic resistant Gram-negative bacteria. Sediment samples were also found to vary more between sites compared to aqueous and biofilm samples. Specifically, the median percentage of bacteria in the aqueous samples resistant to carbapenems (2%) and colistin (10%) at the pristine site (site 1) were similar to the medians for aqueous samples at subsequent sites. In contrast, the sediment samples had higher medians (35% and 20% for carbapenems and colistin respectively) at site 1, which were seen to reduce to a minimum of 9% and 2% respectively for subsequent sites. The aqueous matrix of the river convergence (site 9) showed that 0.6% of bacteria were resistant to gentamicin, while the pristine site, wastewater effluent and treated effluent entry into the environment had a median percentage of gentamicin resistant bacteria of 0%. The sediment showed the median at all these sites was 2%. A higher percentage of sulfamethoxazole resistant bacteria in the sediment compared to aqueous samples was evident at the Eerste/Plankenbrug convergence, and where treated wastewater entered the environment. In the pristine river (colistin; sediment), informal settlement and industrial sites (colistin; aqueous, gentamicin; sediment, and sulfamethoxazole; sediment and biofilm), Eerste/Plankenbrug convergence (all antibiotics; sediment) and the river convergence (carbapenems and colistin; sediment), the maximum value exceeded 100%. In biofilms, the highest median percentage resistance was seen for sulfamethoxazole (10%), followed by colistin, carbapenems and gentamicin. The variation in percentage resistance in biofilm samples to colistin was minimal over the sampling duration.

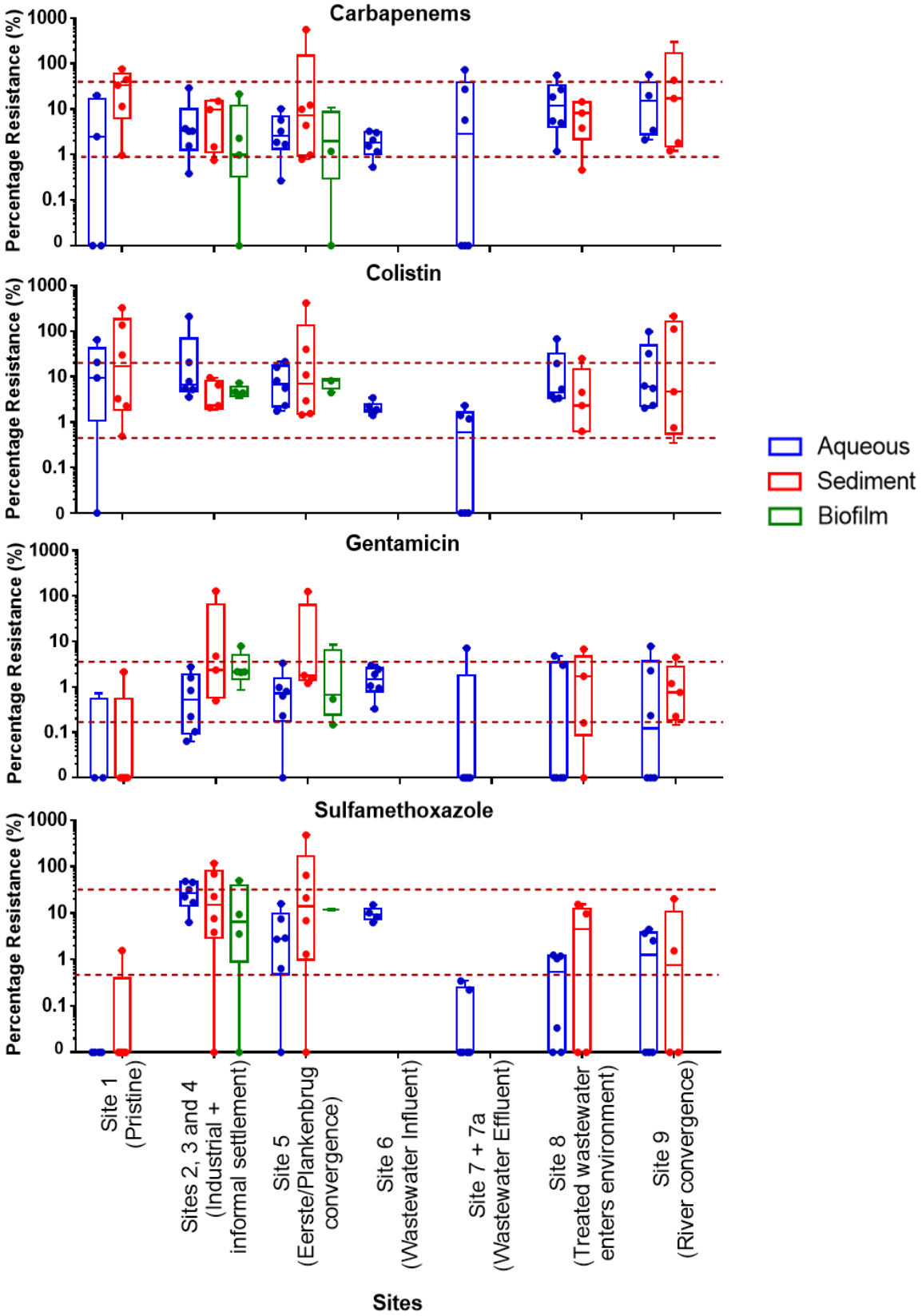

Figure 4.4. Percentage of Gram-negative bacteria that are resistant to carbapenems, colistin, gentamicin and sulfamethoxazole in aqueous, biofilm and sediment river sites, as well as wastewater influent and effluent. Each data point represents a sampling event with the median percentage denoted by the line. Horizontal red dotted lines indicate the range where most medians lie for all sites.

4.3.2. Whole community and single colony genomics

The taxonomical class composition in the aqueous samples varied between months in the river upstream, as well as downstream from the informal settlement and was influenced by industry (Figure 4.5). Bacilli, Bacteroidia, Campylobacteria, and Clostridia were the dominant classes in the latter site, while Gammaproteobacteria was the dominant class for all other sites across all months. The pristine site showed increased abundance of Actinobacteria compared to other sites and Alphaproteobacteria were more dominant in the pristine site and the treated effluent sites compared to the sites impacted by anthropogenic activity.

Pseudomonas spp. and *Aeromonas* spp. were the dominant genera in the carbapenem resistant isolates selected from the SuperCARBA plates (Table 4.1). *Aeromonas* spp. had the highest number of all target antibiotic resistance genes barring for genes conferring resistance to

Figure 4.5. Whole community profiles in aqueous samples based on taxonomical class classification at selected sites and samples months.

multiple antibiotics (multidrug) which were mostly found in *Pseudomonas* spp. Carbapenem resistance genes were seen almost exclusively in *Aeromonas* spp. while peptide, aminoglycoside and sulphonamide/trimethoprim resistance genes were observed over a wider variety of genera.

Table 4.1. Number of target antibiotic resistance genes in each genus identified.

		Number of antibiotic resistance genes by antibiotic class				
Genus	**Number of isolates**	**Carbapenems**	**Peptide**	**Aminoglycoside**	**Sulfonamide /trimethoprim**	**Multidrug***
Pseudomonas	**61**	1	7	37	15	953
Aeromonas	**42**	19	20	79	54	235
Chromobacterium	**6**	0	0	0	0	6
Citrobacter	**4**	0	8	21	10	96
Stenotrophomonas	**4**	0	2	13	3	114
Acinetobacter	**2**	0	0	0	0	20
Comamonas	**2**	0	0	0	0	0
Klebsiella	**2**	0	3	8	1	44
Providencia	**2**	0	0	2	0	23
Burkholderia	**1**	0	0	0	0	3
Chryseobacterium	**1**	0	0	0	0	1
Enterobacter	**1**	1	1	3	0	24
Escherichia	**1**	0	2	4	0	29
Hpunalikevirus	**1**	0	0	2	2	13
Pseudoxanthomonas	**1**	0	0	0	0	4
Rheinheimera	**1**	0	0	0	0	0
Total	**132**	**21**	**43**	**169**	**85**	**1565**

*Resistant to multiple antibiotics

Resistance genes specific for carbapenem resistance were seen in very low numbers in carbapenem resistant isolates (a maximum of 0.5 genes per isolate) (Figure 4.6). These genes were only found at the informal settlement/industrial sites and the Eerste/Plankenbrug convergence, with the most prominent genes being *cphA5*, *cphA7* and *imiH* (Appendix B, Table B1.2). Seven different carbapenem resistance genes were detected across all sites. The number of carbapenem resistance genes in the whole community was also low, with only 2 (*ESP-1* and *cphA7*) carbapenem specific resistance genes observed in the wastewater influent. No carbapenem specific resistant genes were detected in the whole communities at any of the other sites (Figure 4.7). Six different peptide resistance genes (responsible for resistance to colistin) were found in carbapenem resistant isolates (Figure 4.6), with the most prominent being *bacA*, *mdtM* and *mcr-7.1* (Appendix B, Table B1.2) in single colonies and *ICR-Mo, bacA* and *eptA* being the most prominent in whole communities (Appendix B, Table B1.4). Overall, peptide genes, like carbapenem resistance genes were observed in a low abundance (0.5 genes per isolate), however peptide specific genes in whole communities were observed at more sites than carbapenem resistance genes, with the majority being observed in the wastewater influent (Figure 4.7). Aminoglycoside resistance genes (conferring resistance to gentamicin) in single colonies were found at all sites except the pristine site and were the most prominent class of

resistance genes, ranging from 0.25 - 4 genes per isolate (Figure 4.6). Of 24 aminoglycoside resistance genes detected in the carbapenem resistant isolates, *aph(3")-Ib, aph(6)-Id* and *aadA* were the most common (Appendix B, Table B1.2). The number of aminoglycoside resistance genes was higher in the aqueous matrix compared to the biofilm and sediment at all sites, with wastewater influent having the highest average number of genes (4 per isolate for pure cultures and between 30 and 40 genes in whole communities), while wastewater effluent and the river receiving treated effluent had the least (0.25 genes per isolate and less than 5 genes in whole communities). The number of aminoglycoside resistance genes in single colonies was seen to increase at the river convergence. As trimethoprim is used in combination with sulfamethoxazole, genes conferring resistance to trimethoprim were included with sulfamethoxazole resistance genes. *Sul1, dfrA15* and *dfrA14* were the most prominent genes in single colonies (Appendix B, Table B1.2), mostly observed in the aqueous matrix and at the sites influenced by informal settlements and industry, Eerste/Plankenbrug convergence, and wastewater influent in numbers slightly higher than those found for peptides and carbapenems in individual isolates (Figure 4.6). S*ul1, sul2* and *dfrA1* were most prominent in whole communities (Appendix B, Table B1.4) and were mostly found at the informal settlement sites and wastewater influent.

While resistance genes specific to the target antibiotics were detected in the sampling sites, numbers were relatively low compared to multidrug ARG that confer resistance to several antibiotics. 110 different multidrug ARG were detected in carbapenem resistant isolates, of which, *MexB, OpmH and MexK* were the most prominent. Figure 4.6 shows that the highest number of these ARG were found in the aqueous samples at the Eerste/Plankenbrug convergence, where an average of 19 genes were found per isolate. At the informal settlement and industrial sites, and Eerste/Plankenbrug convergence sites where biofilm was collected, it was seen that 10 and 7 genes per isolate were observed respectively in this matrix. At the same sites, sediment was found to contain 7 and 5 genes per isolate, respectively.

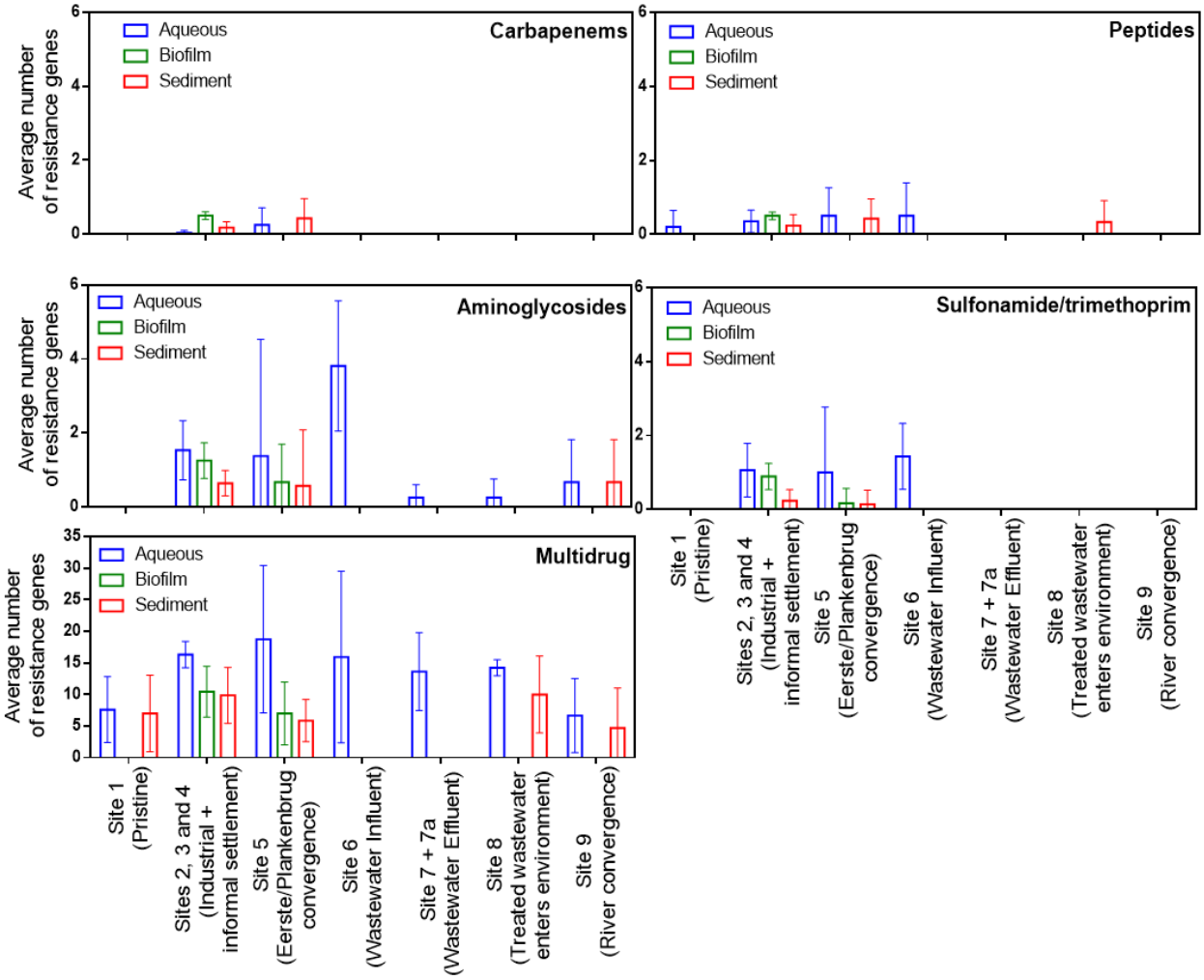

Figure 4.6. Average number of specific, and multidrug ARG detected per isolate in pure cultures isolated on carbapenem media over all sampling events.

Similar to single colonies, multidrug ARG were present in higher numbers compared to target ARG for all sites for whole communities (Figure 4.7). It was observed in both single colonies and whole communities, that the number of target ARG entering the environment post wastewater treatment was reduced compared to the wastewater influent, similar to the reduced percentage of ARB found after wastewater treatment (Figure 4.4). In contrast to the decrease in the percentage of resistant bacteria (Figure 4.3) in the summer months (November-February in SA), the number of resistance genes increased as the temperature increases (Figure 4.7).

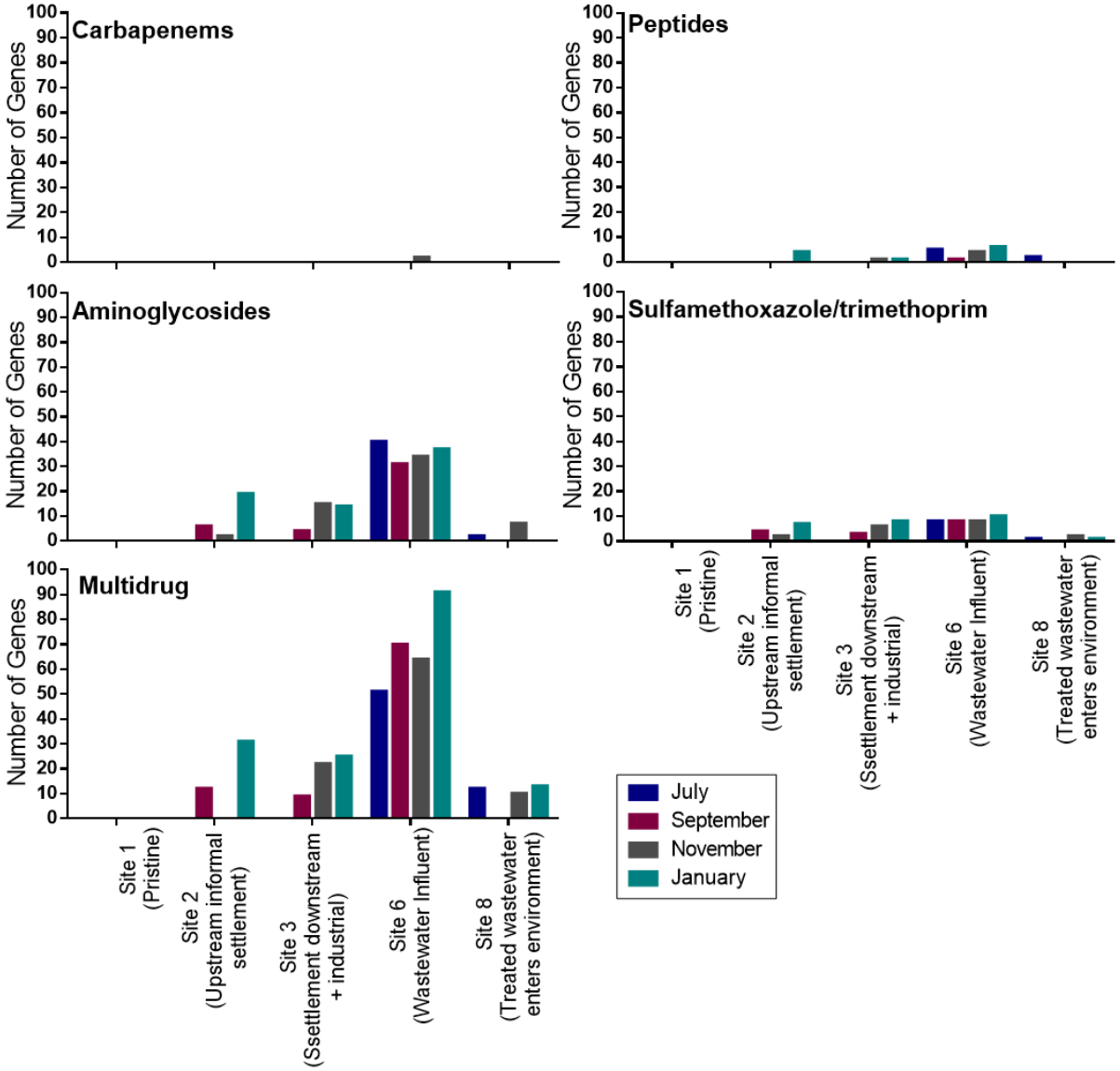

Figure 4.7. Number of target antibiotic resistance genes present at selected sites, identified from whole community aqueous samples.

4.4. Discussion

Data in Figure 4.4 mirrors the fact that gentamicin and sulfamethoxazole are frequently used in a clinical setting for human populations (National Health Laboratory Service, 2013), as a higher median percentage of bacteria resistant to these antibiotics was found at sites in closer proximity to human populations and activities (sites 2 - 6 in Figure 4.2) compared to the other sites that are influenced by agricultural activities. Sulfamethoxazole, in combination with trimethoprim, is used as a prophylactic antibiotic for HIV infected patients (Sibanda et al., 2011; Wininger and Fass, 2002). With 13.5% of the South African population living with HIV in 2019 (Stats SA, 2019), many use co-trimoxazole on a long-term basis. This could lead to the

emergence of co-trimoxazole resistant bacteria at the informal settlements and the residential and hospital settings. Development of resistance in their sectors may influence the Plankenbrug river (sites 2 - 4), Eerste/Plankenbrug convergence (site 5) and wastewater influent (site 6) through improper sanitation at sites 2 through 5, and an aggregation of waste from multiple sectors in the case of site 6.

As a result of its frequent use in a clinical setting, it was expected that the percentage of bacteria resistant to gentamicin would be higher than the observed values (Figure 4.4). Using a concentration of gentamicin more in line with the clinical breakpoint for resistance outlined by the CLSI (Clinical and Laboratory Standards Institute, 2018) to select for resistance in the water samples may have resulted in a similar range of resistant bacteria to the other antibiotics tested. Figures 4.6 and 4.7 suggest that the percentage of bacteria resistant to gentamicin may indeed be misrepresented by using 32 µg/ml to select for these organisms due to the higher number of aminoglycoside resistance genes present in the carbapenem resistant isolates and in whole communities. *Aph(3'')-Ib*, *aph(6)-Id* and *aadA*, amongst other genes have previously been detected in environmental and clinical isolates (Hua et al., 2020). These genes, as well as *sul1*, *mcr* and *cph*, were mostly found in anthropogenically affected sample sites from the isolated colonies (Appendix B, Table B1.2), as well as the whole community profiling (Appendix B, Table B1.4). This suggests that these ARG may originate, or be selected, from human populations and are transferred to environmental waters through bacteria in sewerage, solid waste, and interaction with animals.

Due to the fact that colistin and carbapenems are not readily used in clinical settings yet and have been banned for use in livestock and veterinary purposes, (Woodford et al., 2013), the prevalence of colistin and carbapenem resistance at all sites was unexpected (Figure 4.4). However, resistance due to extensive previous use in livestock could persist and disseminate, leading to the results observed at the pristine site (site 1), entry of treated wastewater into the environment (site 8) and river convergence (site 9), which have agricultural surroundings (Figure 4.2) (European Medicines Agency, 2016; Gouws, 2015). Rain and surface water runoff from these locations could permit resistant organisms entering river waters resulting in resistant bacteria observed at these sites. The most prominent plasmid mediated colistin resistance gene in the pure cultures, *mcr-7.1* (Appendix B; Table B1.2) has also been detected in environmental samples from a zoo, while variations of this gene (*mcr-7*) have been identified in water samples from a recreational club, as well as wastewater, where it was identified as one of the highest occurring *mcr* genes (dos Santos et al., 2020; Furlan et al., 2020; Kneis et al., 2019). *Mcr-7* has

also been found in *K. pneumoniae* isolates obtained from chickens (Yang et al., 2018), while many other *mcr* genes have been identified in clinical isolates worldwide (Rolain et al., 2016; Wise et al., 2018). Notably, colistin resistant isolates have been found in humans and animals that had no previous colistin treatment history, suggesting that resistance may result without the presence of a selective pressure and maintenance of plasmids containing specific resistance genes (Olaitan et al., 2014). Likewise, carbapenem resistant Enterobacteriaceae (CRE) have been found to be prominent in both livestock and wildlife tissue and faecal samples (Glover et al., 2017; Köck et al., 2018). The high number of multidrug resistance genes (most of which are efflux pumps) in both pure cultures and whole communities, in comparison to colistin and carbapenem specific resistance genes (Figure 4.6 and Figure 4.7) may play a role in developing resistance to various antibiotics in the absence of a selective pressure. Efflux pumps are present in all bacterial cells to excrete any unwanted substances. Some of these pumps have non-specific mechanisms of action and thus, although not necessarily evolved to remove antibiotics from the cell, are often present on the chromosome, resulting in intrinsic resistance to multiple antibiotics (Alcalde-Rico et al., 2016). When exposed to low sub-inhibitory antibiotic concentrations, bacterial cells can upregulate the expression of efflux pumps, thus preparing the cell for exposure to higher concentrations that would otherwise be fatal (Rahman et al., 2017). As a result, multidrug resistance genes present in environmental samples may play a greater role in conferring antibiotic resistance in Gram-negative bacteria in the environment compared to target-specific resistance genes. Figure 4.6 shows that carbapenem and multidrug resistance genes in single colonies emerged from predominantly human sources (upstream and downstream from informal settlements and industry, Eerste/Plankenbrug convergence and wastewater influent), while the same observation was made for whole communities (Figure 4.7). ARG were generally found to be reduced in the environment downstream from the treated wastewater discharge.

Colistin resistant *A. baumannii* isolates have been found to grow in the presence of LL-37, a human cationic antimicrobial peptide, that is part of the cathelicidin family of antimicrobial peptides, while colistin susceptible isolates were also susceptible to LL-37. As colistin is also a cationic peptide, exposure of an isolate to certain host responses may play a role in the emergence of resistance (Napier et al., 2013). However, another study did not find any correlation between LL-37 and other cationic antimicrobial peptide (CAMP) resistance and colistin resistance in *E. coli* and *K. pneumoniae* (Dobias et al., 2017). The authors of the latter study indicated that different CAMPs and different species have different responses. Indeed,

animal cathelicidins, in particular BMAP from bovine origin, have been found to be effective in killing colistin resistant Gram-negatives (Kao et al., 2016), as well as pan drug resistant *A. baumannii* (Guo et al., 2018).

The majority of single colonies isolated were *Pseudomonas* spp. and *Aeromonas* spp. (Table 4.1) and are an adequate representation of the whole community as Gammaproteobacteria, to which *Pseudomonas* and *Aeromonas* belong, was the most prominent class across all sites (Figure 4.5). While these genera are predominantly found in water environments (Bhasin et al., 2015; Figueras Salvat and Ashbolt, 2019; Tacão et al., 2015), the selection method used in this study may be a contributing factor to them being detected at a higher frequency than other members of the Gammaproteobacteria class. Some members of *Aeromonas* spp. for example are intrinsically resistant to carbapenems (Tacão et al., 2015). In addition, the *cphA* group of resistance genes confers this resistance to *Aeromonas* spp., while *imiH* has also been identified in *Aeromonas hydrophila* and as such, these genes were among the most commonly detected carbapenem resistance genes in this study (Appendix B, Table B1. 2) (Bello-López et al., 2019; Lim et al., 2019). However, a particular resistance phenotype can be independent of the respective genotype, indicating that merely observing that certain resistance genes were present in an organism does not necessarily mean it would express those genes and display a resistance phenotype to a particular antibiotic (Hughes and Andersson, 2017).

The large monthly variation in the percentage of resistant bacteria to all target antibiotics observed in the wastewater effluent compared to more consistent values from the wastewater influent (Figure 4.4) may be due to several factors. Population shifts during holiday periods, seasonal changes resulting in higher or lower rainfall, which in turn alters flow rates and sporadic pollutants that enter the wastewater system, may affect the treatment efficiency as seen in September, March, and May where ARB were detected opposed to other months, where no ARB were observed in treated effluent (Figure 4.3). SA also experiences scheduled power cuts known as load shedding, which distributes electricity consumption to prevent collapse of the power grid, in which a marked increase in resistant bacteria were observed in the effluent of March and May 2019, when load shedding was implemented (Figure 4.3). Although generators are used in wastewater treatment plants, the delay in the switch from one power source to another may be a contributing factor in the results observed. The prevalence of ARB and ARG were lower in site 8 (treated effluent entering the environment) compared to site 6 (wastewater influent) and other anthropogenically influenced sites (sites 2 - 5), suggesting that

WWTW assisted in reducing the dissemination of ARB into the downstream environment (Figure 4.4 – Figure 4.7).

While individual studies on AMR in aqueous, sediment and biofilm samples have been performed previously, comparative studies between different matrices are limited. In general, the percentage of resistant bacteria in the sediment tended to be higher compared to aqueous or biofilm matrices (Figure 4.4). At some sample sites, the upper limit of the percentage resistance in the sediment and aqueous matrices was greater than 100%. Applying a selective pressure on communities during culturing may have selected certain bacteria that had faster growth rates opposed to antibiotic susceptible species that may have predominated the sample but had a slower growth rate or varied growth requirements, thus resulting in a lower number of bacteria growing on MacConkey agar without supplemented antibiotics (Yang et al., 2017).

Biofilms are known to have an increased tolerance to antibiotics compared to planktonic cells and pose a problem in many aspects of health. For example, antibiotics may not be able to penetrate the biofilm due to the extracellular polymeric substance (EPS) acting as a diffusion-dominated barrier (Ceri et al., 1999; Das et al., 2019; Hughes and Andersson, 2017; Sharma et al., 2019). However, ARG transfer in biofilms is limited in microenvironments within biofilms with genotypically similar cells (Hallatschek et al., 2007), and occurs more prominently in cells on the periphery of the biofilm that have faster metabolic rates (Stalder and Top, 2016). By homogenising the biofilm for plating, persister cells that would have otherwise not been exposed to antibiotics or resistance genes in their natural environment, were exposed to antibiotics in the *in vitro* culturing and thus, biofilms were seen to be more susceptible to antibiotics compared to the other matrices. In future studies, using devices physically placed at the sample site to allow biofilms to attach and mature before being taken to the lab for analysis without disruption may give a better indication of AMR in environmental biofilms.

Aqueous samples, consisting of mostly planktonic cells that do not have EPS and development of associated microenvironments, may be more likely to contain ARB and ARG due to constant influx into river systems and various physiochemical properties that are constantly changing. Shear forces from bulk-liquid allow biofilm erosion, where single or small groups of cells on the biofilm periphery are continuously detached from the matrix, forming part of the aqueous phase before attaching to a new surface (Das et al., 2019). This may have been a contributing factor to the results observed where in some cases the median percentage resistance (Figure

4.4), and the number of ARG in carbapenem resistant isolates (Figure 4.6) were higher in the aqueous phase than in the biofilm and sediment.

4.5. Conclusion

Carbapenem and colistin resistance was found to be more prominent at sites influenced by agriculture compared to gentamicin and sulfamethoxazole, where resistance was found to be more prominent at sites closer to urban anthropogenic activity. Wastewater treatment was found to remove bacteria resistant to most of the antibiotics tested in this study, although large monthly variations were observed in the treated effluent. Single colony ARB and ARG data was found to be representative of whole communities. *Pseudomonas* spp. and *Aeromonas* spp. were the most dominant genera with *cph, imiH, mcr-7.1, bacA, Aph 3''Ib, Aph6-Id, aadA,* and *sul1* amongst the most prominent ARG, however it was suggested that multidrug efflux pumps may play a greater role in environmental antibiotic resistance than specific plasmid mediated ARG. While environmental AMR has been investigated previously, information based on a spatio-temporal approach of multiple sectors of society and matrices is limited. The data discussed in this study indicates the possibility of WWTW limiting the dissemination of environmental AMR and shows the complexity involved when trying to understand the fate of AMR in the environment.

4.6. Acknowledgements

Thank you to the Stellenbosch Wastewater treatment plant and Cape Nature for permitting sampling, Alno Carstens and Ludwig Bröcker for assistance during sampling campaigns and Prof. Daan Nel from the Stellenbosch University Centre for Statistical Consultation for statistical analysis. This work is based on the research supported in part by the National Research Foundation of South Africa (Grant numbers: 118159 and 130527) and the Global Challenges Research Fund (EP/P028403/1).

APPENDIX B

SUPPLEMENTARY MATERIAL FOR CHAPTER 4

Exploring environmental antibiotic resistance in an urban hydrological cycle

B.1. Supplementary results

Table B1. 1. Metadata for single colony genomics

Sample	Species	Sample date	Site	Matrix
A2_1	*Pseudomonas sp. HLS-6*	18/07/2018	Upstream settlement	Aqueous
A2_10	*Pseudomonas fluorescens*	18/07/2018	Convergence town	Aqueous
A2_12	*Aeromonas caviae*	18/07/2018	Wastewater influent	Aqueous
A2_13	*Citrobacter freundii complex sp. CFNIH9*	18/07/2018	Wastewater influent	Aqueous
A2_17	*Pseudomonas alcaligenes*	18/07/2018	Upstream settlement	Aqueous
A2_19	*Pseudomonas sp. HLS-6*	18/07/2018	Convergence town	Aqueous
A2_2	*Pseudomonas sp. HLS-6*	18/07/2018	Downstream settlement	Aqueous
A2_20	*Pseudomonas alcaligenes*	18/07/2018	Treated wastewater in environment	Aqueous
A2_21	*Comamonas kerstersii*	18/07/2018	Final convergence	Aqueous
A2_22	*Pseudomonas stutzeri DSM 10701*	18/07/2018	Wastewater influent	Aqueous
A2_23	*Pseudomonas stutzeri DSM 10701*	18/07/2018	Upstream settlement	Aqueous
A2_24	*Stenotrophomonas maltophilia*	18/07/2018	Wastewater influent	Aqueous
A2_25	*Aeromonas media WS*	18/07/2018	Downstream settlement	Aqueous
A2_26	*Aeromonas media WS*	18/07/2018	Wastewater influent	Aqueous
A2_27	*Aeromonas media WS*	18/07/2018	Wastewater influent	Aqueous
A2_3	*Aeromonas caviae*	18/07/2018	Settlement + industry	Aqueous
A2_30	*Pseudomonas sp. HLS-6*	18/07/2018	Upstream settlement	Aqueous
A2_5	*Pseudomonas sp. HLS-6*	18/07/2018	Settlement + industry	Aqueous
A2_6	*Pseudomonas koreensis*	18/07/2018	Settlement + industry	Aqueous
A2_7	*Pseudomonas protegens*	18/07/2018	Convergence town	Aqueous
A4_11	*Aeromonas sp. ASNIH2*	17/11/2018	Wastewater influent	Aqueous
A4_12	*Aeromonas caviae*	17/11/2018	Wastewater influent	Aqueous
A4_14	*Hpunalikevirus*	17/11/2018	Wastewater influent	Aqueous
A4_15	*Aeromonas caviae*	17/11/2018	Wastewater influent	Aqueous
A4_18	*Pseudoxanthomonas_unclassified*	17/11/2018	Wastewater effluent	Aqueous
A4_2	*Pseudomonas sp. HLS-6*	17/11/2018	Upstream settlement	Aqueous
A4_3	*Pseudomonas sp. HLS-6*	17/11/2018	Upstream settlement	Aqueous
A4_4	*Pseudomonas sp. FGI182*	17/11/2018	Downstream settlement	Aqueous
A4_5	*Stenotrophomonas maltophilia*	17/11/2018	Downstream settlement	Aqueous
A4_7	*Pseudomonas sp. JY-Q*	17/11/2018	Settlement + industry	Aqueous
A4_9	*Pseudomonas putida*	17/11/2018	Convergence town	Aqueous
A5_10	*Citrobacter freundii*	22/01/2019	Convergence town	Aqueous
A5_11	*Citrobacter farmeri*	22/01/2019	Wastewater influent	Aqueous
A5_12	*Aeromonas caviae*	22/01/2019	Wastewater influent	Aqueous
A5_13	*Aeromonas caviae*	22/01/2019	Wastewater influent	Aqueous
A5_14	*Aeromonas caviae*	22/01/2019	Wastewater influent	Aqueous
A5_16	*Pseudomonas putida*	22/01/2019	Treated wastewater in environment	Aqueous
A5_18	*Pseudomonas putida*	22/01/2019	Final convergence	Aqueous
A5_2	*Aeromonas hydrophila*	22/01/2019	Pristine	Aqueous
A5_20	*Pseudomonas putida*	22/01/2019	Upstream settlement	Aqueous
A5_5	*Chromobacterium sp. ATCC 53434*	22/01/2019	Downstream settlement	Aqueous
A5_7	*Pseudomonas sp. JY-Q*	22/01/2019	Settlement + industry	Aqueous
A6_1	*Chromobacterium vaccinii*	25/03/2019	Pristine	Aqueous
A6_10	*Pseudomonas putida*	25/03/2019	Convergence town	Aqueous
A6_11	*Aeromonas caviae*	25/03/2019	Wastewater influent	Aqueous
A6_13	*Stenotrophomonas maltophilia*	25/03/2019	Wastewater effluent	Aqueous
A6_2	*Acinetobacter oleivorans DR1*	25/03/2019	Pristine	Aqueous
A6_5	*Aeromonas hydrophila*	25/03/2019	Downstream settlement	Aqueous
A6_6	*Pseudomonas putida*	25/03/2019	Downstream settlement	Aqueous
A6_7	*Pseudomonas putida*	25/03/2019	Settlement + industry	Aqueous
A6_9	*Aeromonas hydrophila*	25/03/2019	Convergence town	Aqueous

Sample	Species	Sample date	Site	Matrix
A7_1	*Pseudomonas putida*	10/05/2019	Pristine	Aqueous
A7_11	*Citrobacter freundii complex sp. CFNIH9*	10/05/2019	Wastewater influent	Aqueous
A7_12	*Citrobacter farmeri*	10/05/2019	Wastewater influent	Aqueous
A7_13	*Pseudomonas putida*	10/05/2019	Wastewater effluent	Aqueous
A7_14	*Pseudomonas putida*	10/05/2019	Wastewater effluent	Aqueous
A7_15	*Pseudomonas sp. JY-Q*	10/05/2019	Wastewater effluent a	Aqueous
A7_16	*Pseudomonas sp. JY-Q*	10/05/2019	Wastewater effluent a	Aqueous
A7_17	*Pseudomonas putida*	10/05/2019	Treated wastewater in environment	Aqueous
A7_18	*Pseudomonas sp. JY-Q*	10/05/2019	Treated wastewater in environment	Aqueous
A7_19	*Stenotrophomonas maltophilia*	10/05/2019	Convergence all rivers	Aqueous
A7_2	*Pseudomonas putida*	10/05/2019	Pristine	Aqueous
A7_20	*Pseudomonas sp. JY-Q*	10/05/2019	Downstream settlement	Aqueous
A7_3	*Aeromonas caviae*	10/05/2019	Upstream settlement	Aqueous
A7_4	*Shewanella putrefaciens 200*	10/05/2019	Upstream settlement	Aqueous
A7_6	*Pandoraea pnomenusa*	10/05/2019	Downstream settlement	Aqueous
A7_7	*Pseudomonas sp. HLS-6*	10/05/2019	Settlement + industry	Aqueous
A7_8	*Pseudomonas sp. HLS-6*	10/05/2019	Settlement + industry	Aqueous
A7_9	*Pseudomonas sp. HLS-6*	10/05/2019	Convergence town	Aqueous
B2_1	*Aeromonas hydrophila*	18/07/2018	Downstream settlement	Biofilm
B2_10	*Aeromonas salmonicida*	18/07/2018	Downstream settlement	Biofilm
B2_11	*Aeromonas sp. ASNIH4*	18/07/2018	Downstream settlement	Biofilm
B2_12	*Aeromonas salmonicida*	18/07/2018	Downstream settlement	Biofilm
B2_13	*Aeromonas salmonicida*	18/07/2018	Downstream settlement	Biofilm
B2_14	*Aeromonas veronii*	18/07/2018	Downstream settlement	Biofilm
B2_15	*Aeromonas salmonicida*	18/07/2018	Settlement + industry	Biofilm
B2_17	*Pseudomonas koreensis*	18/07/2018	Settlement + industry	Biofilm
B2_18	*Aeromonas salmonicida*	18/07/2018	Downstream settlement	Biofilm
B2_2	*Pseudomonas protegens*	18/07/2018	Downstream settlement	Biofilm
B2_6	*Pseudomonas putida B6-2*	18/07/2018	Settlement + industry	Biofilm
B2_9	*Aeromonas salmonicida*	18/07/2018	Downstream settlement	Biofilm
B4_2	*Aeromonas hydrophila*	17/11/2018	Downstream settlement	Biofilm
B4_3	*Enterobacter sp. Crenshaw*	17/11/2018	Settlement + industry	Biofilm
B4_5	*Comamonas kerstersii*	17/11/2018	Convergence town	Biofilm
B4_6	*Pseudomonas sp. HLS-6*	17/11/2018	Convergence town	Biofilm
B4_7	*Raoultella planticola*	17/11/2018	Downstream settlement	Biofilm
B4_9	*Klebsiella oxytoca*	17/11/2018	Downstream settlement	Biofilm
B5_1	*Chromobacterium sp. ATCC 53434*	22/01/2019	Downstream settlement	Biofilm
B5_5	*Pseudomonas putida HB3267*	22/01/2019	Convergence town	Biofilm
B5_6	*Pseudomonas sp. JY-Q*	22/01/2019	Convergence town	Biofilm
B6_23	*Pseudomonas_putida*	25/03/2019	Settlement + industry	Biofilm
B6_24	*Aeromonas hydrophila*	25/03/2019	Settlement + industry	Biofilm
B6_25	*Aeromonas caviae*	25/03/2019	Convergence town	Biofilm
B6_27	*Pseudomonas alkylphenolica*	25/03/2019	Pristine	Sediment
B6_28	*Chromobacterium vaccinii*	25/03/2019	Pristine	Sediment
B6_29	*Aeromonas hydrophila 4AK4*	25/03/2019	Upstream settlement	Sediment
B6_30	*Aeromonas caviae*	25/03/2019	Upstream settlement	Sediment
B7_21	*Pseudomonas putida H8234*	10/05/2019	Downstream settlement	Biofilm
B7_22	*Pseudomonas sp. HLS-6*	10/05/2019	Downstream settlement	Biofilm
B7_23	*Pseudomonas sp. ATCC 13867*	10/05/2019	Settlement + industry	Biofilm
B7_26	*Aeromonas media WS*	10/05/2019	Convergence town	Biofilm
S2_1	*Pseudomonas putida*	18/07/2018	Downstream settlement	Sediment
S2_15	*Aeromonas rivipollensis*	18/07/2018	Convergence town	Sediment
S2_2	*Aeromonas salmonicida*	18/07/2018	Downstream settlement	Sediment
S2_3	*Pseudomonas sp. HLS-6*	18/07/2018	Convergence town	Sediment
S2_4	*Aeromonas hydrophila*	18/07/2018	Convergence town	Sediment
S2_5	*Pseudomonas fluorescens*	18/07/2018	Convergence town	Sediment
S2_6	*Pseudomonas sp. JY-Q*	18/07/2018	Final convergence	Sediment
S4_10	*Pseudomonas sp. HLS-6*	17/11/2018	Settlement + industry	Sediment
S4_12	*Pseudomonas sp. HLS-6*	17/11/2018	Convergence town	Sediment
S4_14	*Pseudomonas putida*	17/11/2018	Treated wastewater in environment	Sediment
S4_15	*Pseudomonas sp. JY-Q*	17/11/2018	Treated wastewater in environment	Sediment
S4_16	*Chryseobacterium indologenes*	17/11/2018	Final convergence	Sediment

Sample	Species	Sample date	Site	Matrix
S4_19	*Aeromonas veronii*	17/11/2018	Downstream settlement	Sediment
S4_2	*Pseudomonas stutzeri DSM 10701*	17/11/2018	Pristine	Sediment
S4_20	*Citrobacter freundii*	17/11/2018	Downstream settlement	Sediment
S4_3	*Aeromonas caviae*	17/11/2018	Upstream settlement	Sediment
S4_8	*Pseudomonas sp. PONIH3*	17/11/2018	Downstream settlement	Sediment
S4_9	*Pseudomonas sp. ATCC 13867*	17/11/2018	Downstream settlement	Sediment
S5_1	*Chromobacterium vaccinii*	22/01/2019	Pristine	Sediment
S5_11	*Aeromonas hydrophila*	22/01/2019	Treated wastewater in environment	Sediment
S5_14	*Chromobacterium vaccinii*	22/01/2019	Final convergence	Sediment
S5_16	*Aeromonas veronii*	22/01/2019	Settlement + industry	Sediment
S5_17	*Pseudomonas sp. ATCC 13867*	22/01/2019	Upstream settlement	Sediment
S5_2	*Pseudomonas putida W619*	22/01/2019	Pristine	Sediment
S5_4	*Aeromonas caviae*	22/01/2019	Upstream settlement	Sediment
S5_8	*Acinetobacter oleivorans DR1*	22/01/2019	Settlement + industry	Sediment
S5_9	*Aeromonas hydrophila*	22/01/2019	Convergence town	Sediment
S7_29	*Pseudomonas sp. HLS-6*	10/05/2019	Upstream settlement	Sediment
S7_30	*Aeromonas media WS*	10/05/2019	Upstream settlement	Sediment
S7_31	*Pseudomonas sp. ATCC 13867*	10/05/2019	Downstream settlement	Sediment
S7_34	*Pseudomonas sp. HLS-6*	10/05/2019	Settlement + industry	Sediment
S7_36	*Aeromonas hydrophila*	10/05/2019	Convergence town	Sediment

Table B1. 2. Number of antibiotic resistance genes identified in carbapenem resistant isolates for each class of antibiotic at each site.

	Pristine	Settlement/ industrial	Eerste/Plankenbrug Convergence	Wastewater influent	Wastewater effluent	Downstream of treated wastewater into environment	River convergence	Total
Aminoglycoside genes								
aadA	0	14	0	1	0	0	0	26
APH(6)-Id	0	6	0	0	0	0	0	18
APH(3'')-Ib	0	4	0	0	0	0	0	15
arr-3	0	5	0	3	0	0	0	14
aadA2	0	7	1	0	0	0	0	13
arr-2	0	8	0	1	0	0	1	12
acrD	0	7	0	1	0	0	0	12
baeR	0	7	4	6	0	0	1	12
marA	0	7	0	1	0	0	1	12
AAC(6')-Iz	0	3	0	1	1	0	0	6
APH(3')-IIc	0	2	4	7	1	0	1	5
AAC(6')-Ib7	0	0	0	1	0	0	0	3
APH(3')-Ia	0	2	3	9	0	0	0	3
aadA16	0	2	0	1	0	0	0	3
AAC(3)-IIc	0	1	0	6	0	0	0	2
AAC(6')-If	0	1	0	1	0	0	0	2
ANT(2'')-Ia	0	1	1	0	0	0	0	2
aadA22	0	1	1	3	0	0	0	2
aadA5	0	1	2	7	0	0	0	2
golS	0	1	0	0	0	0	0	2
GES-1	0	0	0	0	0	1	0	1
cmlA4	0	1	1	4	0	0	0	1
AAC(6')-Iak	0	0	1	4	0	0	0	0
AAC(6')-Ib	0	0	1	4	0	0	0	0
Carbapenem genes								
cphA5	0	6	0	0	0	0	0	6
cphA7	0	3	0	0	0	0	0	5
imiH	0	1	0	0	0	0	0	4
CEPH-A3	0	2	0	0	0	0	0	2
IMI-3	0	1	0	0	0	0	0	1
cphA2	0	1	2	0	0	0	0	1

	Pristine	Settlement/ industrial	Eerste/Plankenbrug Convergence	Wastewater influent	Wastewater effluent	Downstream of treated wastewater into environment	River convergence	Total
cphA4	0	1	3	0	0	0	0	1
Peptide genes								
bacA	0	7	1	4	0	0	0	12
MCR-7.1	0	5	1	4	0	0	0	12
mdtM	0	5	0	0	0	0	0	10
MCR 3.1	0	4	0	0	0	0	0	4
MCR-3.8	0	2	0	0	0	0	0	2
MCR-3.6	1	1	5	0	0	1	0	1
Sulfonamide/trimethoprim genes								
sul1	0	19	0	0	0	0	0	33
dfrA15	0	9	1	7	0	0	0	13
dfrA14	0	3	1	3	0	0	0	11
sul2	0	3	1	0	0	0	0	5
dfrA12	0	4	0	1	0	0	0	4
dfrA17	0	1	0	0	0	0	0	2
dfrA23	0	1	0	0	0	0	0	2
dfrA5	0	2	0	0	0	0	0	2
dfrB1	0	1	0	1	0	0	0	1
dfrB4	0	1	4	10	0	0	0	1
sul4	0	0	1	1	0	0	0	1
Multidrug genes								
MexB	5	39	15	6	5	7	2	79
OpmH	4	40	16	6	5	6	2	79
mexK	6	38	15	6	5	6	2	78
Pseudomonas aeruginosa CpxR	5	38	15	6	5	6	2	77
MexE	5	38	15	5	5	7	2	77
MexF	5	38	15	6	5	6	2	77
mexW	4	36	12	5	5	6	2	70
MuxB	8	30	10	4	5	6	3	66
TriC	4	27	13	5	5	6	2	62
CRP	1	34	9	16	0	1	0	61
OprN	5	27	10	4	5	6	2	59
OprM	4	24	9	5	5	6	2	55
OXA-12	1	26	8	11	0	1	0	47

	Pristine	Settlement/ industrial	Eerste/Plankenbrug Convergence	Wastewater influent	Wastewater effluent	Downstream of treated wastewater into environment	River convergence	Total
arnA	1	27	14	2	0	1	0	45
MexD	2	10	3	3	2	3	0	23
MexA	2	13	4	1	0	3	0	23
mexI	0	11	3	2	2	2	1	21
PmpM	1	14	3	1	0	1	0	20
oqxB	0	9	1	3	2	1	1	17
KPC-1	0	3	2	11	0	0	0	16
TEM-1	0	5	1	7	0	0	0	13
cpxA	0	7	1	4	0	0	0	12
emrA	0	7	1	4	0	0	0	12
emrB	0	7	1	4	0	0	0	12
mdtA	0	7	1	4	0	0	0	12
mdtC	0	7	1	4	0	0	0	12
mdtH	0	7	1	4	0	0	0	12
pmrF	0	7	1	4	0	0	0	12
acrB	0	7	1	4	0	0	0	12
emrR	0	7	1	4	0	0	0	12
msbA	0	7	1	4	0	0	0	12
yojI	0	7	1	4	0	0	0	12
ramA	0	7	1	4	0	0	0	12
H-NS	0	7	1	4	0	0	0	12
tolC	0	7	1	4	0	0	0	12
kdpE	0	6	1	4	0	0	0	11
Pseudomonas aeruginosa soxR	0	7	2	1	0	1	0	11
mdsB	0	5	0	4	1	1	0	11
baeS	0	5	1	4	0	0	0	10
MexC	0	6	1	1	0	2	0	10
smeD	0	5	0	2	2	0	1	10
smeE	0	5	0	2	2	0	1	10
smeF	0	5	0	2	2	0	1	10
eptA	0	4	1	4	0	0	0	9
acrE	0	4	1	4	0	0	0	9
mdtB	0	6	1	2	0	0	0	9
sdiA	0	4	1	4	0	0	0	9
Escherichia coli acrA	0	4	1	3	0	0	0	8

	Pristine	Settlement/ industrial	Eerste/Plankenbrug Convergence	Wastewater influent	Wastewater effluent	Downstream of treated wastewater into environment	River convergence	Total
OprJ	0	6	1	1	0	0	0	8
OpmB	0	6	1	1	0	0	0	8
mexJ	0	6	1	1	0	0	0	8
Klebsiella pneumoniae OmpK37	0	4	0	4	0	0	0	8
smeA	0	4	0	1	1	0	1	7
smeB	0	4	0	1	1	0	1	7
smeC	0	4	0	1	1	0	1	7
smeR	0	4	0	1	1	0	1	7
smeS	0	4	0	1	1	0	1	7
MOX-6	0	1	0	6	0	0	0	7
MuxA	0	5	1	1	0	0	0	7
MuxC	0	5	1	1	0	0	0	7
Pseudomonas aeruginosa emrE	0	3	2	1	0	1	0	7
acrF	0	3	1	2	0	0	0	6
oqxA	0	4	0	2	0	0	0	6
FOX-5	0	5	0	0	0	0	0	5
MOX-5	0	4	1	0	0	0	0	5
mexL	0	3	1	1	0	0	0	5
mexV	0	3	1	1	0	0	0	5
MOX-9	0	1	1	2	0	0	0	4
ugd	0	2	0	2	0	0	0	4
msrE	0	1	1	2	0	0	0	4
Sed1 beta-lactamase	0	1	0	2	0	0	0	3
PDC-1	0	1	1	0	0	1	0	3
vgaC	0	1	0	2	0	0	0	3
NDM-1	0	1	0	1	0	0	0	2
GES-9	0	1	1	0	0	0	0	2
FOX-4	0	2	0	0	0	0	0	2
MOX-7	0	0	0	2	0	0	0	2
OXA-1	0	1	0	1	0	0	0	2
abeM	1	1	0	0	0	0	0	2
Enterobacter cloacae acrA	0	1	0	1	0	0	0	2
Klebsiella pneumoniae acrA	0	2	0	0	0	0	0	2
adeF	1	1	0	0	0	0	0	2
adeG	1	1	0	0	0	0	0	2

	Pristine	Settlement/ industrial	Eerste/Plankenbrug Convergence	Wastewater influent	Wastewater effluent	Downstream of treated wastewater into environment	River convergence	Total
adeH	1	1	0	0	0	0	0	2
abeS	1	1	0	0	0	0	0	2
adeI	1	1	0	0	0	0	0	2
adeJ	1	1	0	0	0	0	0	2
adeK	1	1	0	0	0	0	0	2
adeN	1	1	0	0	0	0	0	2
TEM-110	0	1	0	1	0	0	0	2
ACT-10	0	1	0	0	0	0	0	1
NmcR	0	1	0	0	0	0	0	1
GES-14	0	0	0	1	0	0	0	1
SHV-2	0	0	0	1	0	0	0	1
CGB-1 beta-lactamase	0	0	0	0	0	0	1	1
FOX-9	0	1	0	0	0	0	0	1
MOX-2	0	1	0	0	0	0	0	1
MOX-3	0	0	0	1	0	0	0	1
OKP-B-5	0	1	0	0	0	0	0	1
OXA-101	0	0	0	1	0	0	0	1
OXA-332	1	0	0	0	0	0	0	1
OXA-357	0	1	0	0	0	0	0	1
OXA-47	0	1	0	0	0	0	0	1
OXA-62	0	1	0	0	0	0	0	1
ceoB	0	1	0	0	0	0	0	1
mdtF	0	1	0	0	0	0	0	1
mexG	0	1	0	0	0	0	0	1
VEB-3	0	1	0	0	0	0	0	1
OXY-1-3	0	1	0	0	0	0	0	1
TEM-150	0	0	1	0	0	0	0	1
TEM-168	0	0	0	0	0	0	0	0

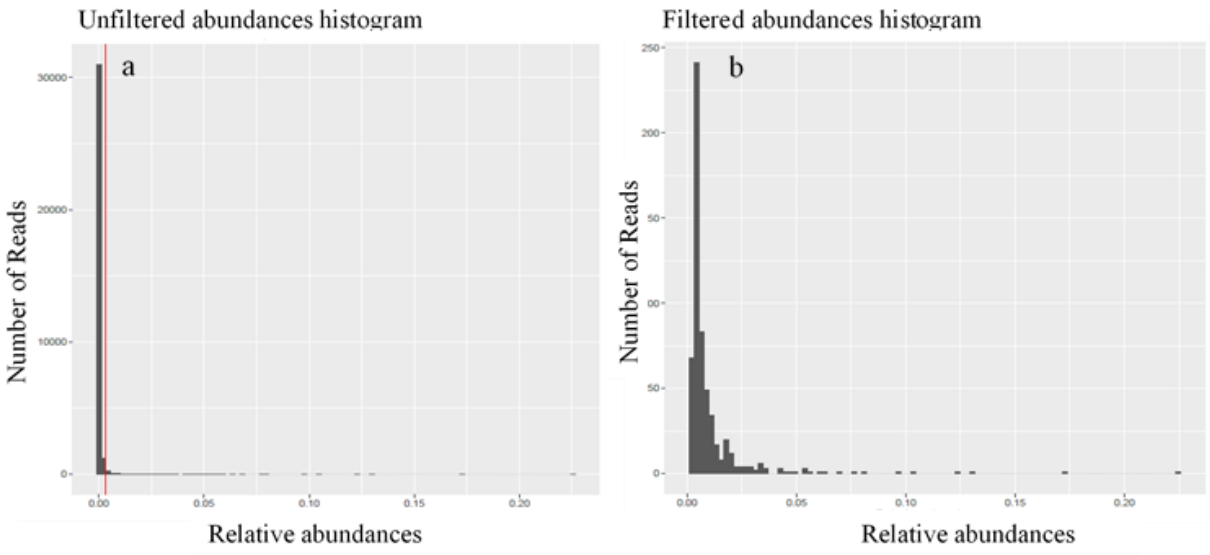

Figure B1.1. Abundance of taxonomical classes in whole communities before (a) and after (b) filtering out low abundance reads at a threshold of 0.003%.

Table B1. 3. Metadata for whole community genomics

Sample name	# Bases	# Bases - post trimming	# Reads	# Reads - post trimming	% Duplicates	mean read length	median read length	mode read length	Sample Date	Site	Matrix	Volume filtered (ml)	DNA concentration (ng/ul)
A2_6	9,830,865,294	9,344,512,356	39,166,794	39,165,432	1.7	238.2	251	251	19/07/2018	Wastewater influent	Aqueous	250	61.3
A2_8	56,499,901,710	42,406,539,347	225,099,210	225,086,800	1.2	187.2	251	251	18/07/2018	Treated wastewater into the environment	Aqueous	1000	4.4
A3_2	21,309,144,992	16,822,236,198	84,896,992	84,884,304	0.3	197.4	251	251	19/09/2018	Upstream informal settlement	Aqueous	250	12.8
A3_3	8,595,781,642	8,336,568,989	34,246,142	34,245,108	1.5	243.3	251	251	19/09/2018	Downstream informal settlement	Aqueous	500	29.5
A3_6	11,448,494,030	10,876,311,882	45,611,530	45,609,838	1.2	238.1	251	251	20/09/2018	Wastewater influent	Aqueous	150	24.4
A4_1	6,898,629,078	6,692,965,212	27,484,578	27,483,698	2.7	243.4	251	251	17/11/2018	Pristine	Aqueous	1000	13.4
A4_2	6,806,437,782	6,678,658,197	27,117,282	27,116,052	1	246.2	251	251	17/11/2018	Upstream informal settlement	Aqueous	500	71.8
A4_3	7,944,049,098	7,740,793,752	31,649,598	31,648,334	1.8	244.4	251	251	17/11/2018	Downstream informal settlement	Aqueous	350	4.8
A4_6	7,615,196,428	6,132,658,820	30,339,428	30,337,396	0.2	200.8	251	251	18/11/2018	Wastewater influent	Aqueous	150	94.3
A4_8	8,188,321,796	8,001,007,092	32,622,796	32,622,188	2.7	245.3	251	251	17/11/2018	Treated wastewater into the environment	Aqueous	1000	26.3
A5_2	7,854,620,810	7,487,452,636	31,293,310	31,292,144	1.3	239.1	251	251	22/01/2019	Upstream informal settlement	Aqueous	150	31.3
A5_3	8,332,827,014	7,757,431,031	33,198,514	33,197,304	1.1	233.1	251	251	22/01/2019	Downstream informal settlement	Aqueous	210	26.5
A5_6	8,369,642,690	8,148,419,949	33,345,190	33,344,562	1.5	244.1	251	251	23/012019	Wastewater influent	Aqueous	100	26.9
A5_8	7,923,649,324	7,737,774,422	31,568,324	31,567,768	2.1	245.1	251	251	22/01/2019	Treated wastewater into the environment	Aqueous	1000	10.6

Table B1. 4. Antibiotic resistance genes in whole communities from aqueous samples

	Pristine	Settlement upstream	Settlement + industry downstream	Wastewater influent	Downstream of treated wastewater into environment	Total
Aminoglycosides						
APH(3'')-Ib	0	1	1	1	1	4
APH(6)-Id	0	1	1	1	0	3
aadA3	0	1	1	1	0	3
aadA5	0	1	1	1	0	3
aadA	0	0	1	1	0	2
aadA2	0	0	1	1	0	2
ANT(3'')-IIa	0	0	0	1	1	2
aadA22	0	0	1	1	0	2
aadA24	0	1	0	1	0	2
aadA13	0	1	0	1	0	2
aadA6/aadA10	0	1	0	1	0	2
AAC(6')-Ib-Suzhou	0	0	1	1	0	2
aadS	0	1	0	1	0	2
aadA10	0	0	0	1	1	2
aadA7	0	0	1	1	0	2
AAC(6')-Ib'	0	0	0	1	1	2
AAC(6')-Ib9	0	0	0	1	0	1
ANT(2'')-Ia	0	0	0	1	0	1
aadA12	0	0	0	1	0	1
AAC(6')-IIa	0	0	0	1	0	1
AAC(6')-Ib	0	0	0	1	0	1
AAC(6')-Ib-Hangzhou	0	0	0	1	0	1
aadA11	0	0	0	1	0	1
aadA27	0	0	0	1	0	1
aadA4	0	0	0	1	0	1
AAC(6')-Ib4	0	0	1	0	0	1
ANT(3'')-IIc	0	0	0	1	0	1
aadA16	0	0	0	1	0	1
aadA17	0	0	0	1	0	1
AAC(3)-Ia	0	0	0	1	0	1
AAC(6')-Ib10	0	0	0	1	0	1
AAC(6')-Ib8	0	0	0	1	0	1

	Pristine	Settlement upstream	Settlement + industry downstream	Wastewater influent	Downstream of treated wastewater into environment	Total
ANT(3'')-Ii-AAC(6')-IId fusion protein	0	0	0	1	0	1
APH(3')-IIIa	0	0	0	1	0	1
aadA15	0	0	0	1	0	1
aadA6	0	0	0	1	0	1
aadA8	0	0	0	1	0	1
acrD	0	0	0	1	0	1
AAC(3)-IId	0	0	0	1	0	1
AAC(6')-Ib7	0	0	0	1	0	1
APH(2'')-IVa	0	1	0	0	0	1
AAC(6')-Ir	0	0	0	1	0	1
ANT(6)-Ia	0	0	0	1	0	1
AAC(6')-Ib11	0	1	0	0	0	1
aadA21	0	0	1	0	0	1
Carbapenems						
ESP-1	0	0	0	1	0	1
cphA7	0	0	0	1	0	1
Peptides						
ICR-Mo	0	1	0	1	0	2
bacA	0	0	0	1	0	1
eptA	0	0	0	1	0	1
pmrF	0	1	0	0	0	1
Sulfonamides/trimethoprim						
sul1	0	1	1	1	1	4
sul2	0	1	1	1	0	3
dfrA1	0	1	1	1	0	3
dfrA14	0	1	1	1	0	3
dfrB5	0	0	1	1	0	2
dfrA19	0	0	1	0	0	1
dfrG	0	0	0	1	0	1
dfrA16	0	1	0	0	0	1
dfrA22	0	1	0	0	0	1
dfrA27	0	1	0	0	0	1
dfrA32	0	0	0	1	0	1
dfrF	0	0	0	1	0	1

	Pristine	Settlement upstream	Settlement + industry downstream	Wastewater influent	Downstream of treated wastewater into environment	Total
dfrA26	0	0	1	0	0	1
dfrA5	0	0	0	1	0	1
dfrA7	0	0	1	0	0	1
Multidrug						
msrE	0	1	1	1	1	4
MexB	0	0	1	1	1	3
Pseudomonas aeruginosa CpxR	0	1	0	1	1	3
MexF	0	1	0	1	1	3
marA	0	1	0	1	1	3
MuxB	0	0	0	1	1	2
mexK	0	0	0	1	1	2
mexW	0	0	0	1	1	2
mel	0	0	1	1	0	2
CRP	0	0	1	1	0	2
H-NS	0	0	1	1	0	2
adeK	0	1	0	1	0	2
gadX	0	1	0	1	0	2
Klebsiella pneumoniae KpnF	0	1	1	0	0	2
Enterobacter cloacae acrA	0	0	1	1	0	2
MexE	0	0	0	1	1	2
baeR	0	1	0	1	0	2
oqxA	0	1	0	1	0	2
oqxB	0	0	1	1	0	2
tetX	0	1	0	1	0	2
Klebsiella pneumoniae OmpK37	0	0	1	1	0	2
VEB-3	0	1	1	0	0	2
ErmB	0	0	0	1	0	1
ErmF	0	0	0	1	0	1
adeJ	0	0	0	1	0	1
tolC	0	0	0	1	0	1
evgA	0	1	0	0	0	1
lsaE	0	0	0	1	0	1
mtrA	0	1	0	0	0	1
ErmG	0	0	0	1	0	1
mdtE	0	0	0	1	0	1

	Pristine	Settlement upstream	Settlement + industry downstream	Wastewater influent	Downstream of treated wastewater into environment	Total
acrB	0	0	0	1	0	1
Klebsiella pneumoniae acrA	0	0	1	0	0	1
ceoB	0	0	0	0	1	1
cpxA	0	0	0	1	0	1
AAC(6')-Ib-cr	0	0	0	1	0	1
Escherichia coli acrA	0	1	0	0	0	1
ramA	0	0	1	0	0	1
MOX-9	0	0	0	1	0	1
VEB-9	0	1	0	0	0	1
acrE	0	0	0	1	0	1
mdtO	0	1	0	0	0	1
mdtP	0	0	0	1	0	1
OXA-10	0	0	1	1	0	2
OXA-101	0	0	1	1	0	2
OXA-280	0	0	0	1	1	2
Escherichia coli ampH	0	1	0	1	0	2
Klebsiella pneumoniae OmpK37	0	0	1	1	0	2
OXA-464	0	0	1	1	0	2
OXA-58	0	1	0	1	0	2
OXA-296	0	1	0	1	0	2
OXA-17	0	0	0	1	0	1
OXA-427	0	0	0	1	0	1
OXA-663	0	0	0	1	0	1
OXA-11	0	0	0	1	0	1
OXA-2	0	0	0	1	0	1
OXA-211	0	0	0	1	0	1
OXA-233	0	0	0	1	0	1
OXA-246	0	0	0	1	0	1
OXA-333	0	0	0	1	0	1
Laribacter hongkongensis ampC beta-lactamase	0	0	0	1	0	1
NPS-1	0	0	0	1	0	1
OXA-142	0	0	0	1	0	1
OXA-212	0	0	0	1	0	1
OXA-256	0	0	0	1	0	1
OXA-368	0	0	0	1	0	1

	Pristine	Settlement upstream	Settlement + industry downstream	Wastewater influent	Downstream of treated wastewater into environment	Total
OXA-373	0	0	0	1	0	1
OXA-96	0	0	0	1	0	1
OXA-12	0	1	0	0	0	1
OXA-129	0	0	1	0	0	1
OXA-14	0	0	0	1	0	1
OXA-240	0	0	0	1	0	1
OXA-74	0	0	0	1	0	1
Escherichia coli ampC	0	0	0	1	0	1
GES-24	0	0	1	0	0	1
OXA-278	0	1	0	0	0	1
OXA-3	0	0	0	1	0	1
VEB-2	0	0	0	0	1	1

CHAPTER 5

SUB-MINIMUM INHIBITORY CONCENTRATIONS OF ZINC CHLORIDE AND SULFAMETHOXAZOLE-TRIMETHOPRIM PROMOTE PERSISTER CELL DEVELOPMENT IN BIOFILMS

Abstract

The increased use of heavy metals in various applications has led to the concern that heavy metal resistance may co-select for antibiotic resistance mechanisms, thus increasing the development of antimicrobial resistance (AMR). Biofilms are known to resist antibiotic treatment and as a result, the effect of biofilm exposure to heavy metals needs to be investigated. This study measured carbon dioxide (CO_2) production as an indication of biofilm metabolism in real time to determine the effects of exposure to MIC_{50} concentrations of sulfamethoxazole-trimethoprim (SMX:TMP) and zinc chloride ($ZnCl_2$) on the metabolic activity of biofilms and how exposed biofilms respond to various concentrations of SMX:TMP. Susceptibility of planktonic cells and biofilms to SMX:TMP and $ZnCl_2$ was determined by microbroth dilutions. Microbial community shifts and diversity were determined using automated ribosomal intergenic spacer analysis (ARISA), and fluorescence microscopy was performed to determine the relative abundance of live and dead cells in biofilms at different treatment points. Initial exposure to MIC_{50} concentrations of SMX:TMP and $ZnCl_2$ resulted in biofilm metabolic activity below the detection limit of the system until the treatments were removed, after which metabolic profiles were similar to the metabolic profiles of biofilms that did not have initial antimicrobial treatment. Microscopy suggested that initial MIC_{50} SMX:TMP exposure resulted in a microbial community that consisted of species that may be strong biofilm formers, while biofilms initially exposure to MIC_{50} $ZnCl_2$ appeared to consist of species that were weak biofilm formers. Diversity amongst microbial communities shifted after antibiotic treatments. Biofilms initially treated with antimicrobials had a less diverse community compared to the control biofilm. It was concluded that exposure to MIC_{50} concentrations of SMX:TMP and $ZnCl_2$ did not increase overall resistance to SMX:TMP in the biofilms but rather resulted in the development of persister cells and antibiotic- and heavy metal tolerant cells within the biofilm.

5.1. Introduction

Antimicrobial resistance (AMR) is perpetuated by inappropriate prescription and disposal of antibiotics, as well as widespread use in agricultural settings for prophylactic purposes, and growth promotion in livestock (Baynes et al., 2016; Ventola, 2015). Biofilms, which are sessile microbial communities, are notoriously more resistant to antibiotics compared to their planktonic counterparts (Harrison and Turner, 2007). These communities are problematic both in an environmental, and medical setting by causing water quality issues in pipelines, and through causing persistent infections, as treatment of biofilms with antibiotics has little to no effect on biofilm formation (Sahoo et al., 2015; Sharma et al., 2019). Heavy metals including copper, nickel, silver, gold, aluminium, cobalt, zinc, and others can have antimicrobial properties at high enough concentrations with a greater antimicrobial effect on biofilms compared to antibiotics. As a result, they are applied therapeutically, and in research on nanoparticle synthesis (Harrison et al., 2004; Turner, 2017). However, biofilms have been shown to be tolerant to lower concentrations of these metals. As a result, biofilms have been implemented for bioremediation of heavy metals that occur naturally in the environment or as waste products through industrial processes and agriculture (Gurbanov and Severcan, 2020). It has been proposed that tolerance to heavy metals co-select for AMR due to metal resistance genes and antibiotic resistance genes (ARG) being present on the same mobile genetic elements resulting in concern for the selection of antibiotic resistant bacteria (ARB) that further reduces treatment options. In addition, cross resistance may occur whereby a bacterial cell has a mechanism of action that confers resistance to a metal, as well certain antibiotics. Efflux pumps are the most common example (Baker-Austin et al., 2006).

Sulfamethoxazole-trimethoprim (SMX:TMP) antibiotic combinations are frequently prescribed to HIV positive patients, as well as tuberculosis (TB) patients for prophylactic reasons. Due to a large proportion of South Africans living with HIV and TB, this antibiotic combination is highly relevant (Stats SA, 2019). In addition, *sul1,* a gene conferring resistance to sulfamethoxazole is often found in the environment (dos Santos et al., 2020; Heuer and Smalla, 2007; Obayiuwana et al., 2018; Suzuki et al., 2015), suggesting the potential for co-selection or cross resistance with heavy metal resistance genes to occur. Zinc oxide (ZnO) was frequently used to supplement pig feed to prevent diarrhoea in piglets at concentrations more than 2250 mg/kg (Zhang et al., 2020). As $ZnCl_2$ is soluble in water while ZnO is not, $ZnCl_2$'s toxicity in the environment is reduced and as a result, has become more widely used in a number of applications (PubChem: ZnCl2, n.d.; PubChem: ZnO, n.d.). Most studies evaluating

the effect of antibiotics and heavy metals on biofilms involved single species biofilms, and at therapeutic dose concentrations (Harrison et al., 2004, 2005; Zhang et al., 2020). In contrast, this study aimed to evaluate the effect of sub-inhibitory concentrations of SMX:TMP and $ZnCl_2$ on mixed-culture biofilms by using CO_2 production as an indication of biofilm metabolism, and subsequently identifying SMX:TMP and $ZnCl_2$ susceptibility to identify potential co- or cross-resistance between these antimicrobials.

5.2. Materials and Methods

5.2.1. Sampling and inoculum preparation

A 50 ml grab sample of return activated sludge (RAS) was collected from a wastewater treatment facility in Cape Town, SA. Samples were transported on ice, 1 ml aliquots were made with 40% glycerol final volume and then frozen at -30°C until needed in all subsequent experimentation.

To prepare the biofilm inoculum, frozen aliquots were thawed on ice and inoculated into 1% Tryptone Soya Broth (TSB) at room temperature and incubated overnight at 37°C with aeration.

5.2.2. Determination of MIC and MIC_{50} concentrations

Microbroth dilutions were performed in quadruplicate in 96 well plates with doubling dilutions of concentrations (Andrews, 2001) ranging from 16 - 512 µg/ml for $ZnCl_2$, as well as SMX:TMP in a 19:1 ratio to replicate the optimal synergistic ratio *in vivo* as stipulated by the CLSI (Smilack, 1999; Clinical and Laboratory Standards Institute, 2018). Each well contained 77.6 µl Mueller Hinton Broth (MHB), the appropriate volume of each treatment to obtain the correct concentration in a final volume of 200 µl, and PBS to make up a volume of 180 µl. Overnight RAS culture described above was diluted with sterile PBS to obtain a McFarland standard equivalent (optical density at 600 nm: 0.08-0.12) and 20 µl of this dilution was inoculated into each well to make the final well volume 200 µl. Positive control wells contained no $ZnCl_2$ or SMX:TMP, and negative control wells contained 20 µl PBS instead of inoculum.

Microtiter plates were incubated with shaking at 37 °C overnight and optical densities were measured using an iMark™ Microplate Absorbance Reader (BioRad) at 595 nm. Absorbance readings for each concentration of each treatment were divided by the average absorbance reading of the positive control to obtain the proportion of growth that each treatment concentration had in relation to the positive control. The resulting values were plotted, and the concentrations that corresponded to a proportion of 0.5 (indicative of the concentration that

inhibits 50% of the organism's growth (MIC_{50})) were determined by interpolation. The resulting concentrations were considered sub-MIC and used for the initial treatment of the biofilm metabolism experiments.

5.2.3. Biofilm studies

Biofilms were cultivated in a CO_2 evolution measurement system (CEMS) as previously described (Jackson et al., 2015; Kroukamp and Wolfaardt, 2009). This system uses CO_2 analysers to detect CO_2 produced by biofilm respiration as an indication of biofilm metabolism in real time.

5.2.3.1. CEMS reactor set up

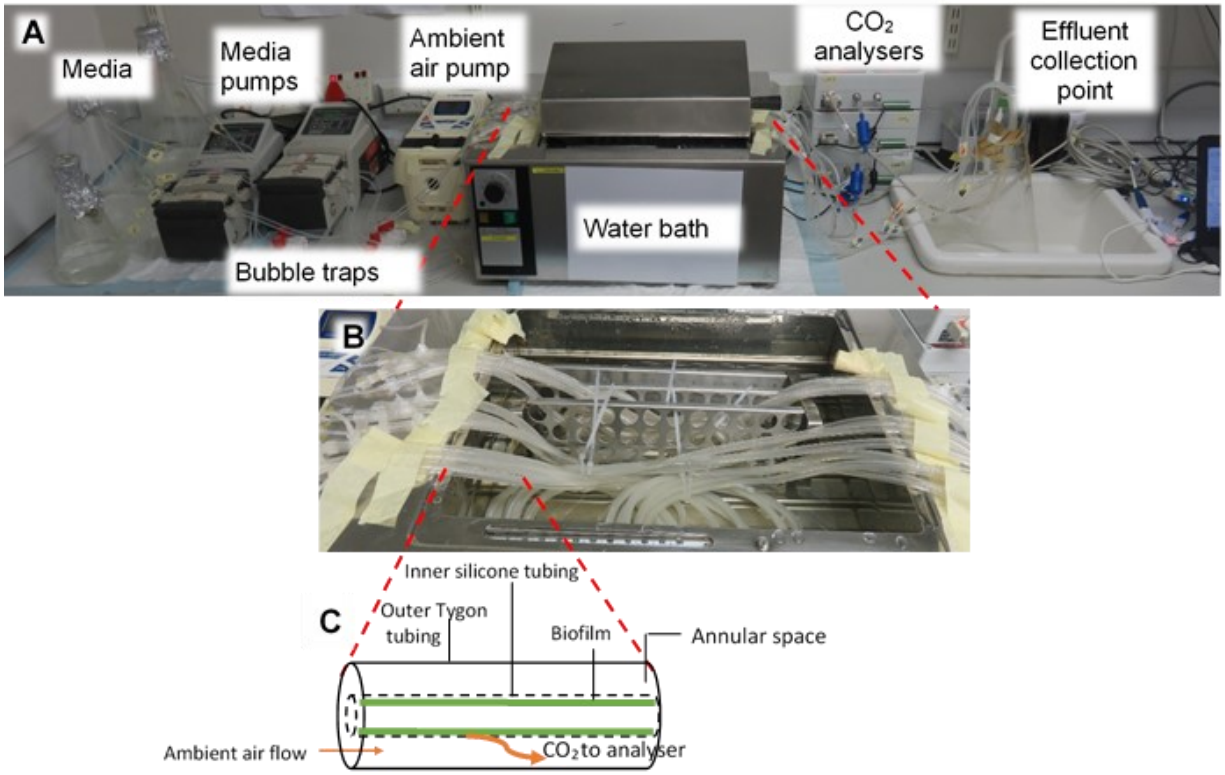

Figure 5.1. CO_2 evolution measurement system (CEMS) set up for biofilm metabolism monitoring in real time. A) The reactor in its entirety, B) the CEMS component of the reactor where the biofilm is cultivated, submerged in the water bath, and where dilution rates and retention times are calculated, and C) a schematic diagram of the CEMS principle.

In this system (Figure 5.1), 160 cm gas-permeable silicone tubing (1.6 mm internal diameter, 2.4 mm outer diameter) was placed within 150 cm gas-impermeable Tygon tubing (4.8 mm internal diameter, 7.9 mm outer diameter). CO_2 produced as a result of biofilm growth within the silicone tubing diffused out of the silicone tubing and into the annular space, which was then passed through CO_2 analysers (LI-820, LI-COR Biosciences) with ambient air as sweeper

gas at a flow rate of 9 ml/h. CEMS tubing was submerged in a water bath to keep a constant temperature of 37 °C ± 1°C (Figure 5.1B). Calculations to convert CO_2 measurements from ppm to µmol/h are shown in Appendix C.1. Bubble traps were connected in series after the peristaltic pumps (Watson-Marlow 205S) to prevent the formed biofilms from being disrupted by air bubbles. All other tubing connecting the media to the CEMS tubing and effluent collection point was silicone tubing (internal diameter 1.6 mm, outer diameter, 3.2 mm).

5.2.3.2. Growth conditions and treatments

The reactor was sterilised with 10% commercial bleach solution for 2 h, followed by rinsing with sterile dH_2O overnight. Subsequently, media harbouring different treatment conditions (i-biofilms established in absence of an antimicrobial (0.3 g/L TSB), ii-SMX:TMP (0.3 g/L TSB + RAS culture MIC_{50} concentration determined above (646:34 µg/ml) SMX:TMP, iii- $ZnCl_2$ (0.3 g/L TSB + RAS culture MIC_{50} concentration (51 µg/ml) $ZnCl_2$)) was continuously fed through the silicone tubing (13 ml/h) until the reactor volume had been displaced. The media pump was then stopped and 300 µl overnight RAS culture prepared as described above was injected (25G needle) into the silicone tubing immediately before the Tygon tubing for each treatment. The hole left by the needle was sealed with marine silicone sealant (Bostik). The pump was left off for 45 min to allow inoculated cells to attach to the silicone tubing before continuing the media flow. The biofilm response to each treatment was performed in triplicate, however, due to the limited number of CO_2 analysers, CO_2 was only measured from one biofilm exposed to each treatment. Planktonic cells produced by the biofilms (medium displacement rate greatly exceeded maximum growth rates) were routinely sampled at the effluent collection point shown in Figure 5.1A. Biofilm samples were obtained by sacrificing one of the silicon-tube biofilm reactors for each condition at various time points. Before sacrificing the respective biofilms, the sweeper gas was switched between the biofilm reactors to determine the reproducibility of the CO_2 profiles between replicates. Treatment conditions and concentrations varied with time depending on the CO_2 profiles that emerged and are provided in Table 5.1. A total of 6 repeats were performed for each treatment.

Table 5.1. Concentrations of antimicrobials (µg/ml) added to 0.3 g/L TSB during experimentation for each biofilm growth condition.

Biofilm growth condition	Biofilm establishment			Determination of effect of lower concentrations of treatment	Effect of sub-MIC SMX:TMP	Biofilm recovery	Effect of sub-MIC SMX:TMP after recovery
(i)	No AM*				456:24 SMX:TMP†	No AM	608:32 SMX:TMP††
(ii)	646:34 SMX:TMP	228:12 SMX:TMP**	No AM	133:7 SMX:TMP***	456:24 SMX:TMP†	No AM	608:32 SMX:TMP††
(iii)	51 $ZnCl_2$	40 $ZnCl_2$**	No AM	20 $ZnCl_2$***	20 $ZnCl_2$+ 456:24 SMX:TMP†	No AM	608:32 SMX:TMP††

*No antimicrobial
**MIC_{50} determined from planktonic cell yield in the first 24 h of biofilm development in condition (i).
***MIC_{25} determined from planktonic cell yield after 144 h of biofilm development in condition (i).
†Double the lowest SMX:TMP concentration that had an inhibitory effect.
††MIC_{50} determined from planktonic cell yield at 209 h of biofilm development in condition (i).

5.2.3.3. Reactor sample collection and enumeration of viable cells

5.2.3.3.1. Planktonic cells

Samples were collected daily in falcon tubes (between 10 and 20 ml depending on biomass present) from the effluent collection point of all 9 reactors (3 treatments in triplicate) shown in Figure 5.1A.

5.2.3.3.2. Biofilms

Biofilms from each treatment type were sacrificed at different time points (after CO_2 profile reached steady state for all treatments, after all lines were spiked with 456:24 µg/ml SMX:TMP concentrations, and at the end of the experiment after the final treatment with 608:32 µg/ml SMX:TMP) following the protocol by Jackson et al., 2019. Briefly, bulk liquid was collected in 50 ml falcon tubes and 0.1 M NaOH, pre-heated to 60°C was used to disrupt loosely attached biofilm. Two ml NaOH was injected into the silicone tubing and incubated at 60 °C for 30 min, to dislodge firmly attached cells after which, the remaining biofilm was squeezed out of the tubing and excess cells were flushed out with PBS. Biomass was pelleted, washed once with PBS to remove excess NaOH and resuspended in PBS. While NaOH is disruptive to bacterial cells, biofilms are able to withstand antimicrobial effects of chemicals and high temperatures more so than their planktonic counterparts (Sharma et al., 2019). In addition, Corcoran et al. (2014) established that 1M NaOH had very little effect on eradicating 168 h *Salmonella* spp. biofilms. As a result, it was deemed the potential effect of 0.1 M NaOH on biofilm viability was negligible opposed to the advantage of improved disruption of biofilm from the tubing.

Planktonic and biofilm samples collected from all 9 reactors were serially diluted in PBS, plated on 3 g/L TSA plates in duplicate, and incubated at 37 °C for 18-24 h for enumeration.

5.2.3.4. Susceptibility testing

MICs of the planktonic effluent samples were performed in duplicate as described above, however the concentration ranges differed ($ZnCl_2$ (16 - 512 µg/ml) and SMX:TMP (304:16 – 1216:64 µg/ml) for the first repeat and $ZnCl_2$ (2 - 64 µg/ml) and SMX:TMP (304:16 – 1216:64 µg/ml) in the second repeat). Where the absorbance of the effluent sample at 600 nm was less than the McFarland standard turbidity range (0.08 - 0.12), 4 ml of samples were centrifuged at 10000 ×g for 2 min and resuspended in 1 ml PBS to concentrate.

5.2.4. Analysis of bacterial diversity

Automated ribosomal intergenic spacer analysis (ARISA) was performed on the second repeat of the experiment to determine if the various treatments resulted in a bacterial community shift. Depending on the biomass present in the sample, between 2- and 8-ml planktonic samples were collected from the effluent collection point in triplicate for each treatment at the following time points; when the CO_2 profile first reached steady state, after the biofilm recovered following spiking of all lines with 456:24 µg/ml SMX:TMP, and at the end of the experiment. Samples from sacrificed biofilms were also used for ARISA analysis. Samples were spun at 10000 ×g for 5 min and resuspended in 200 µl PBS. DNA extraction was performed using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (ZymoResearch) according to manufacturer's recommendations. DNA samples were sent to Sporatec (https://www.sporatec.co.za) for ARISA analysis. Briefly, fluorescently labelled primers were used (ITSReub: GCCAAGGCATCCACC and ITSf - FAM labelled: GTCGTAACAAGGTAGCCGTA) to amplify the 16S rRNA regions with PCR in triplicate. Resulting amplicons were analysed on an ABI 3010XL Genetic analyser. The peaks from the produced electropherograms were converted to fragment lengths according to Slabbert et al., 2010.

5.2.5. Microscopy

Planktonic cells collected at 66 h and 234 h, at intervals as described in section 5.2.3.3.1 were harvested by centrifugation and washed with PBS. Cell pellets were resuspended in 1 ml saline and the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen) was used to stain the cells. 2 µl SYTO™ 9 and 2 µl propidium iodide (PI) was added to 1 ml saline. One hundred µl of the prepared BacLight stain was added to the 1 ml sample, mixed well, and incubated in the dark at room temperature for 30 min. Centrifugation at 10000 ×g for 2 min was performed to

remove unbound stain and the pellet was washed with saline. Cells were observed using a Nikon Eclipse TE2000-E inverted fluorescence microscope using a blue laser (420 - 495 nm) for excitation of both fluorophores. SYTO™ 9 resulted in green fluorescence while PI resulted in red fluorescence. Where green cells were not observed, cells were observed using a green laser (510 - 560 nm) which only excites PI. Images were captured using a Canon EOS 550D camera and post-processing was done using ImageJ software.

5.3. Results

5.3.1. Determination of MIC_{50}

Both $ZnCl_2$ and SMX:TMP at 64 µg/ml completely inhibited growth of the mixed RAS culture. Proportion of growth at the varying treatment concentrations was established relative to the optical density of the positive control (no treatment). Interpolated MIC_{50} values were 51 µg/ml and 646:34 µg/ml respectively, as shown by the dotted red lines in Figure 5.2. These concentrations were used as the initial treatment concentrations for biofilms (ii) and (iii), respectively in the CEMS experiments as indicated in Table 5.1.

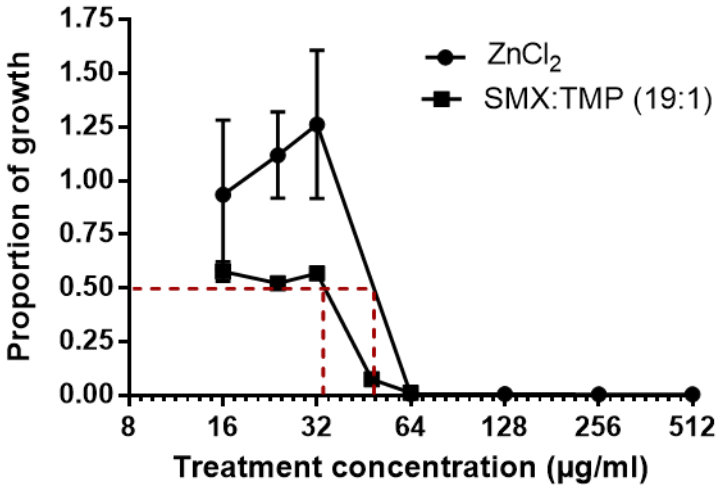

Figure 5.2. Proportion of the growth of an overnight return activated sludge culture in different SMX:TMP and $ZnCl_2$ concentrations to determine their respective MIC_{50}s. For the SMX:TMP combination, the TMP concentration is plotted. The dotted red lines indicate the interpolated concentrations at which 50% of the population was inhibited.

5.3.2. Whole-community biofilm CO_2 profiles

The technical replicates (line 1-green and 2-blue) in each experiment observed in each frame in Figure 5.3 mostly showed similarity in CO_2 production to the main CO_2 profile (line 3-black). In addition, the CO_2 profiles showed smilar trends between biological repeats (Figure 5.3 A - C and Figure 5.3 D - E). The viable cells from all sacrificed biofilms had similar trends.

The repeats between the CO_2 profiles of biofilm (i) (no initial antimicrobial exposure; Table 5.1) showed a notable difference after 96 h, with the first experiment showing decreased CO_2 production, while the biofilm in the second experiment continued to gradually increase CO_2 production. After inoculation, there was an exponential increase in biofilm development, lasting 14 h (Figure 5.3 A and D), with varied further increase and decrease until the 456:24 µg/ml SMX:TMP treatment at 160 h. The CO_2 produced by the biofilm sharply reduced to 2.5 and 3 µmol/h in the first and second repeats respectively (Figure 5.3 A and D). Removing the 456:24 µg/ml SMX:TMP treatment at 188 h showed an immediate increase in CO_2 production (Figure 5.3 A and D), followed by a reestablishment of steady state. Treatment with 608:32 µg/ml SMX:TMP resulted in rapid decrease in CO_2 production to below the detection limit. Planktonic cell yield for biofilm (i) remained relatively constant throughout the first experiment duration (between 8.1×10^7 and 1.7×10^8 CFU/ml) (Figure 5.3 A) and numbers observed in the second experiment were roughly an order of magnitude higher (Figure 5.3 D).

Despite the sub-MIC (MIC_{50}) concentrations of SMX:TMP identified in Figure 5.2 being used initially, biofilm (ii) (Table 5.1) did not show any visible CO_2 production after inoculation, even when the concentration of the treatment was reduced at 20 h to 228:12 µg/ml SMX:TMP (Figure 5.3 B and E). CO_2 was exponentially produced at 72h once the initial treatment had been removed after 65.5 h with steady state reached at 82 h (Figure 5.3 B and E). When spiked with a lower concentration of the SMX:TMP combination (133:7 µg/ml) at 125 h, a rapid increase in CO_2 production to 34 µmol/h was observed and a new steady state established. Upon exposure to 456:24 µg/ml SMX:TMP, the CO_2 produced by biofilm (ii) was reduced to 12 µmol/h. As with biofilm (i), removing the 456:24 µg/ml SMX:TMP treatment at 188 h showed an increase in CO_2 production (Figure 5.3 B and E), followed by a reestablishment of steady state. Treatment with 608:32 µg/ml SMX:TMP resulted in a rapid decrease in CO_2 production to 2 µmol/hr in the second repeat (Figure 5.3 E). Planktonic cell yield was in the range of 10^3 CFU/ml before 72 h and after the initial treatment was removed, viable planktonic cells reached similar numbers to that observed in biofilm (i).

As with biofilm (ii) (initial exposure to MIC_{50} SMX:TMP), biofilm (iii) did not detect any CO_2 production at initial sub-MIC $ZnCl_2$ concentrations, as well as when the concentration was reduced to 40 µg/ml (Figure 5.3 C and F). When the $ZnCl_2$ treatment was removed, CO_2 was produced exponentially and reached steady state at 90 h and 111 h in the first (Figure 5.3 C) and second (Figure 5.3 F) experiments respectively, although the CO_2 profiles fluctuated extensively. The lag phase in the repeated experiment (Figure 5.3 F) was longer than the lag

phase in the first (Figure 5.3 C) after the initial $ZnCl_2$ treatment was removed. CO_2 production was inhibited immediately upon exposure to 20µg/ml $ZnCl_2$ at 120 h, and the subsequent addition of 456:24 µg/ml SMX:TMP did not change the CO_2 profile. Treatment with 608:32 µg/ml SMX:TMP resulted in a rapid decrease in CO_2 production to below the detection limit as with biofilm (ii). No planktonic cell production for $ZnCl_2$ treated biofilms was evident for the first experiment until the initial treatment was removed at 65.5 h (Figure 5.3 C), and the second experiment showed approximately 90 CFU/ml at 20 h (Figure 5.3 F). Here, the biofilm had 10^2 CFU/ml at 91 h (while the CO_2 profile was still in lag phase). Once CO_2 production reached steady state at 117 h, viable cell counts in biofilm (iii) were similar to those of initially untreated biofilms (biofilm (i)). Planktonic cell yield decreased in biofilm (iii) when CO_2 production decreased due to exposure to treatments and increased when the treatments were removed, and CO_2 production increased. The second experiment resulted in a lower number of viable cells (10^2 CFU/mm^2) in biofilm (iii) (Figure 5.3 F) compared to the first (Figure 5.3 C). Viable planktonic cells in biofilm (iii) decreased after 608:32 µg/ml SMX:TMP exposure (Figure 5.3 C).

.

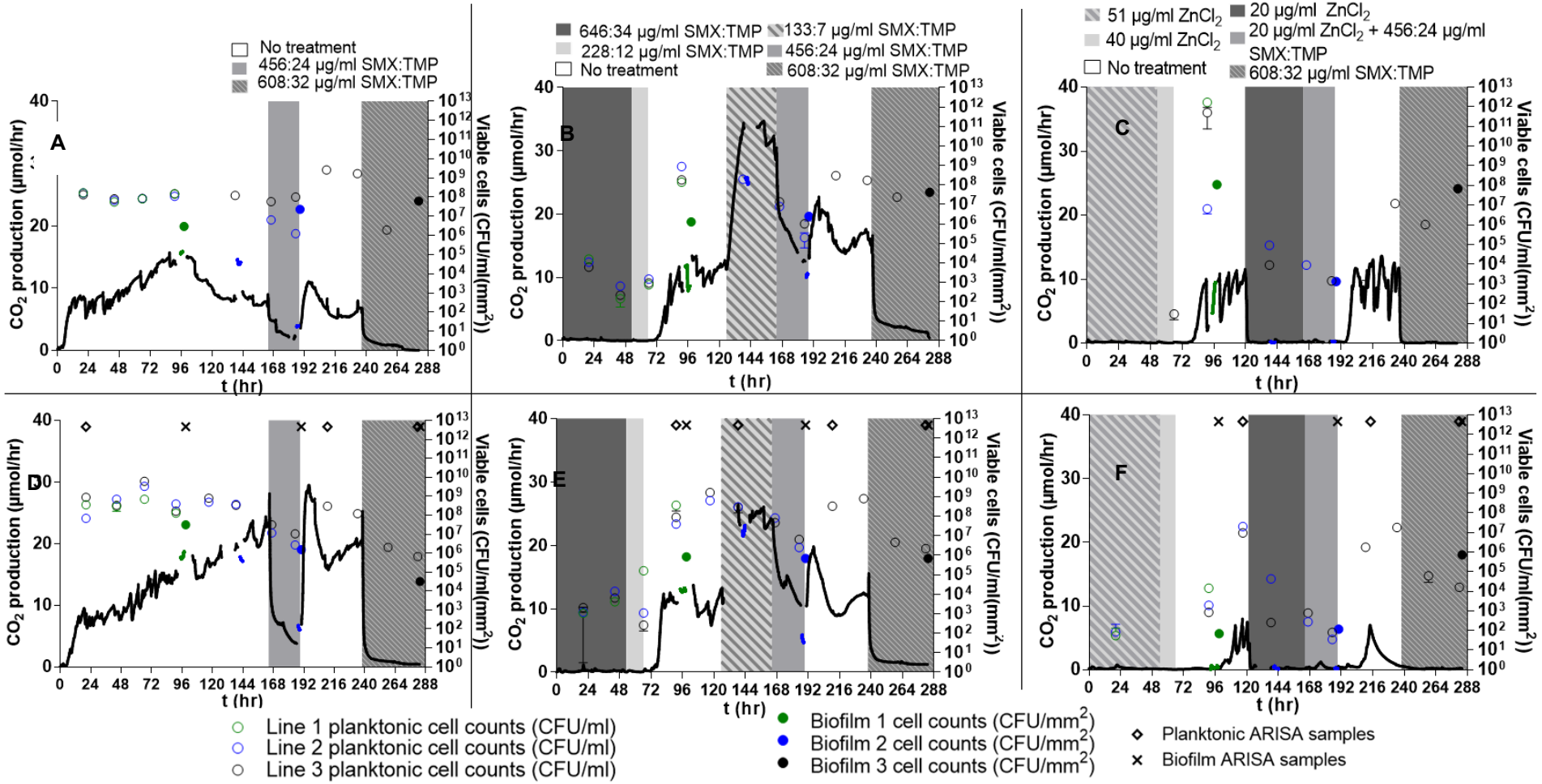

Figure 5.3. Real time CO_2 production profiles and planktonic and biofilm viable cell counts from return activated sludge biofilms cultivated in a CO_2 Evolution Measurement System (CEMS). Biofilms were exposed to different treatment conditions selected based on the MIC_{50} values of the initial RAS culture, and from the planktonic cells identified during the experiment and are listed in Table 5.1. The CO_2 profile was primarily tracked in one reactor (black line), with intermittent comparisons between reactors (as described in section 5.2.3.2) indicated in green (repeat 1) and blue (repeat 2). Frames D-F are a repeated experiment of frames A-C. A, D – biofilm (i) (no initial treatment), B, E- biofilm (ii) (initial exposure to MIC_{50} of SMX:TMP), C, F – biofilm (iii) (initial exposure to MIC_{50} of $ZnCl_2$). The time points at which ARISA samples were taken for the repeated experiment are annotated on D-F. Malfunctioning of the laptop-interphase resulted in the loss of data between 127 h and 137 h seen in D - F. Planktonic plate counts were enumerated as CFU/ml sample collected from the reactor effluent, while biofilm biomass present in the silicone tubing was calculated to yield CFU/mm^2.

5.3.3. SMX:TMP and $ZnCl_2$ MICs of viable planktonic and harvested biofilm cells

The MICs of the RAS culture used for inoculation of the CEMS system (t_0) were 1216:48 µg/ml and 64 µg/ml for SMX:TMP and $ZnCl_2$ respectively for experiment 1 (Figure 5.4 A - C), and 912:48 µg/ml and 32 µg/ml for SMX:TMP and $ZnCl_2$ respectively for experiment 2 (Figure 5.4 D - E). The MIC occurring the most often (mode MIC) for the rest of the time points was 456:24 µg/ml for SMX:TMP and 64 µg/ml for $ZnCl_2$ in the first experiment for all treatment conditions, and the mode MIC in the second experiment for SMX:TMP was 912:48, 304:16 and <152:8 µg/ml, and for $ZnCl_2$ was 16, 32 and 16 µg/ml for biofilms (i), (ii), and (iii) respectively. The first experiment showed an increase in SMX:TMP MIC in treatment biofilm (i) and (ii) biofilms upon exposure to 456:24 µg/ml SMX:TMP at 165.5 h, while the second experiment showed a decrease in SMX:TMP MICs. MICs to $ZnCl_2$ decreased for all biofilm treatment conditions at this time point (Figure 5.4 A - F). Viable cells harvested from the sacrificed biofilms had similar $ZnCl_2$ MICs to planktonic cells at the respective time points in all treatments, however the SMX:TMP MICs differed between biofilm and planktonic cells. The MICs for both SMX:TMP and $ZnCl_2$ in the biofilms upon termination of the experiment were higher in biofilms (ii) and (iii) compared to the biofilm (i) for the first experiment, however the opposite is true for the second experiment.

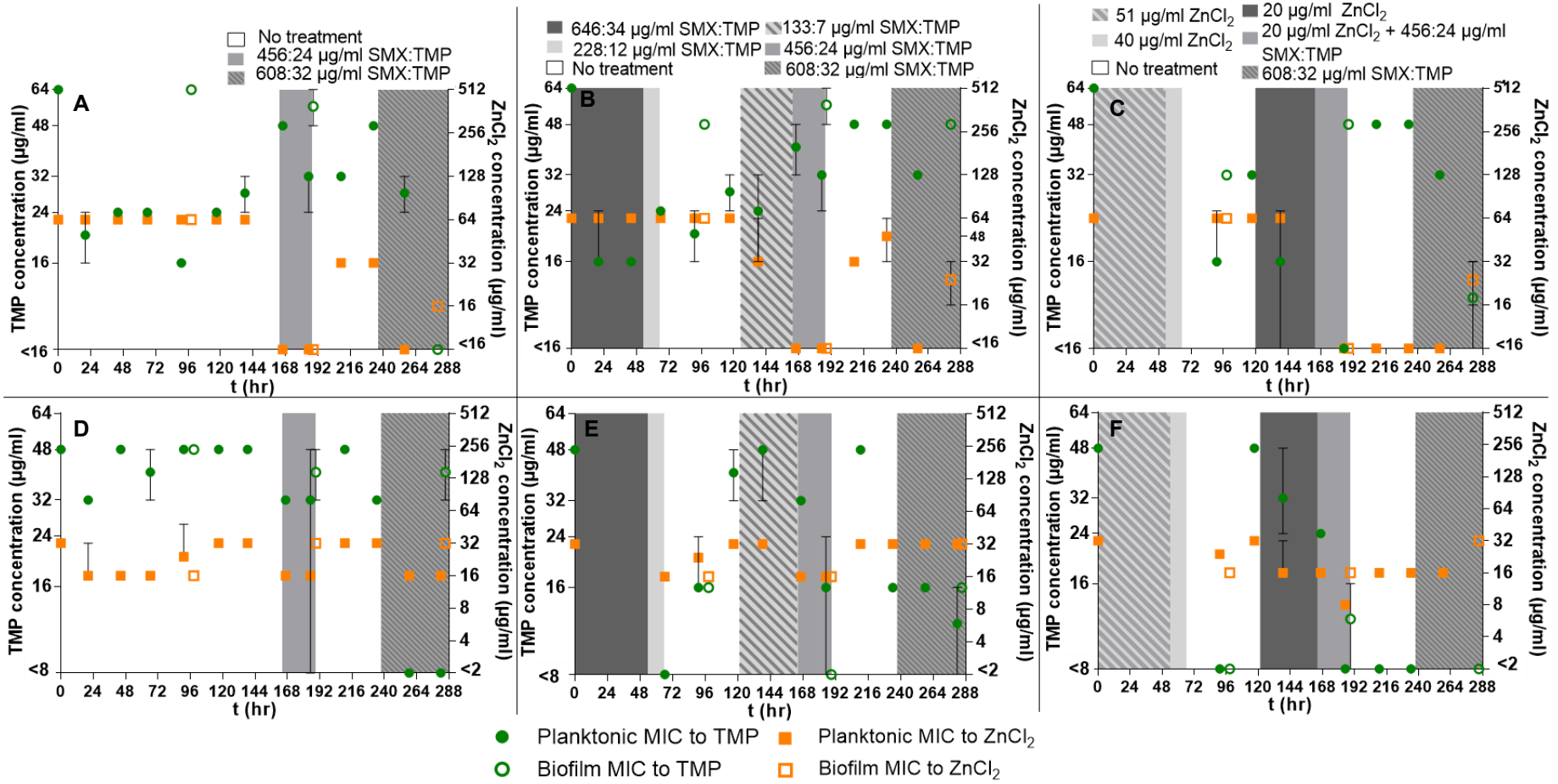

Figure 5.4. Median minimum inhibitory concentrations of biofilm and planktonic cell yield from cultivated biofilms after various treatments of SMX:TMP and ZnCl2. Only the TMP concentration is shown on the left y-axis for the 19:1 SMX:TMP combination. D-F are repeats of A-C, respectively. A, D – biofilm (i), B, E – biofilm (ii), C, F – biofilm (iii). Error bars indicate the maximum and minimum MICs from two repeats.

5.3.4. Analysis of bacterial diversity

The exponential Shannon and inverse Simpson indices (Figure 5.5) indicate the bacterial diversity and evenness within the samples at given time points after different treatments. Diversity profiles between the planktonic cells and biofilm were similar. Whole community biofilms (ii) and (iii) were seen to be less diverse (indicated by low alpha diversity values) compared to the biofilms that were not exposed to any treatment during biofilm (i) establishment. The bacterial diversity and evenness in biofilm (i) biofilms increased upon the first exposure to SMX:TMP (Figure 5.5 B and D), which became more dominant when the CO_2 production in the biofilm reached the new steady state at approximately 216 h (Figure 5.3 D), as indicated by the lower indices in the planktonic cells at steady state after treatment (Figure 5.5 A and C). SMX:TMP spikes had little effect on the microbial diversity of biofilms (ii) and (iii), however slight increased diversity was observed in these biofilms after the final SMX:TMP treatment (Figure 5.5 B).

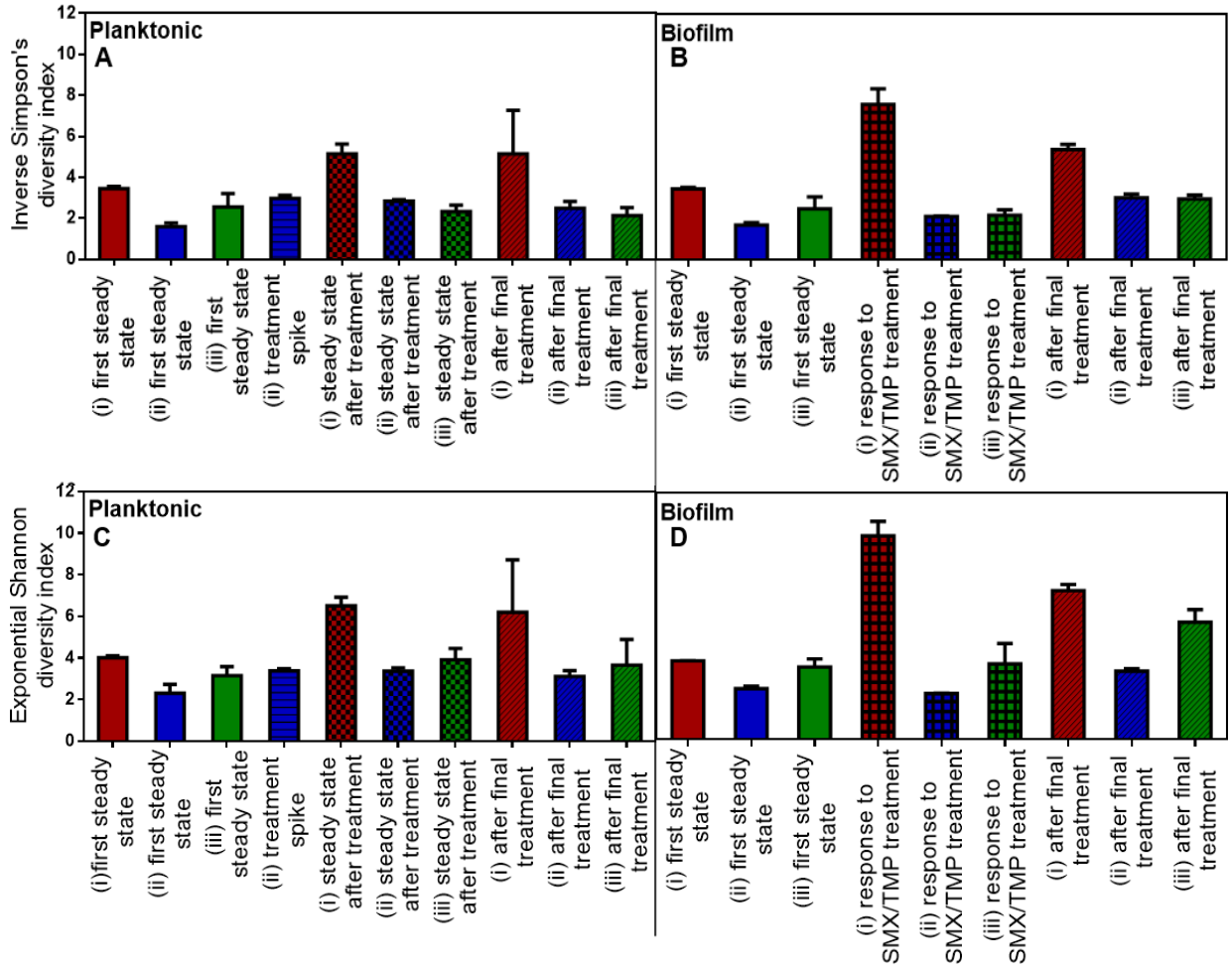

Figure 5.5. Alpha diversity indices indicating diversity and evenness of bacterial communities in planktonic and biofilm samples after each stage of the respective treatment conditions as described in Table 5.1. Higher values represent higher diversity and evenness.

The beta diversity NMDS plot shows that microbial communities between the planktonic and biofilm samples were similar for each treatment condition at the first steady state, however communities for the different treatment conditions were distinctly different. Communities in biofilm (ii) were seen to be unrelated to biofilms (i) and (iii) in both biofilm and planktonic samples (Figure 5.6). The microbial community in biofilm (iii) were similar at all time points and between planktonic and biofilm cells. It was expected that biofilm (ii) would have overlapping ellipses indicating similar community composition after the antibiotic combination spikes, however this was not the case in the planktonic cell yield. Instead, biofilms (ii) and (i) showed more distinct differences in community composition from the first steady state after the CO_2 profiles recovered from the first SMX:TMP spike (Figure 5.3 D - F at 216 h). Biofilms (i) and (ii) had different community structures in response to the 456:24 µg/ml spike (Figure 5.6). After the final 608:32 µg/ml SMX:TMP treatment, all biofilms ((i), (ii), and (iii)) had different community compositions compared to those present at the first CO_2 steady state.

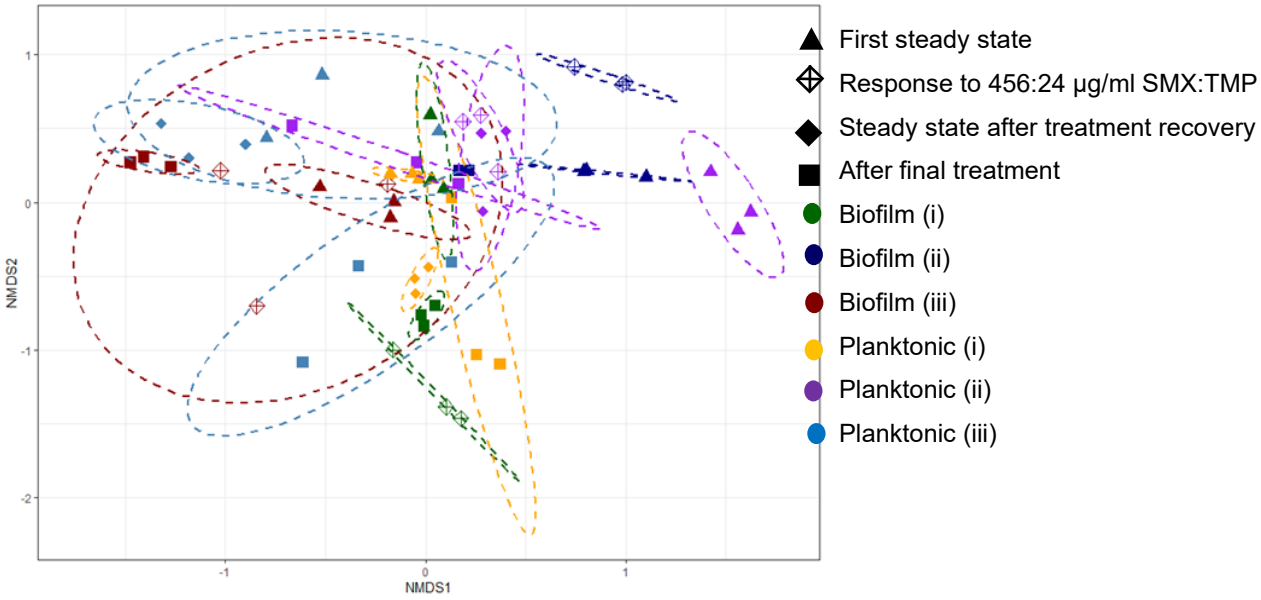

Figure 5.6. Beta diversity of biofilm and planktonic communities after each stage of the respective treatment conditions as described in Table 5.1. Symbols of similar shape and colour are replicates.

5.3.5. Microscopy

Upon removal of the initial MIC_{50} SMX:TMP and $ZnCl_2$ treatments from biofilms (ii) and (iii) respectively at 66h, planktonic cell yield from the biofilms was evident for all treatments (Figure 5.7 A - C), despite no planktonic $ZnCl_2$ treated viable cells being enumerated at this time point (Figure 5.3 F). All cells fluoresced green and as a result were deemed live. The cells from biofilm (i) were more distinct whereas the biofilms (ii) and (iii) appeared to have a

cloudier appearance. After recovery from the 456:24 µg/ml SMX:TMP spike, biofilm (i) (Figure 5.7 D) had very few cells despite multiple efforts to obtain a better image, however cells that were observed, were green. Biofilms (ii) and (iii) were found to have red cells (dead cells) in addition to green cells (live cells) in the planktonic cell yield after the biofilms had recovered from the SMX:TMP spike. The planktonic cells observed in biofilm (ii) appeared to be more clustered compared to biofilms (i) and (iii).

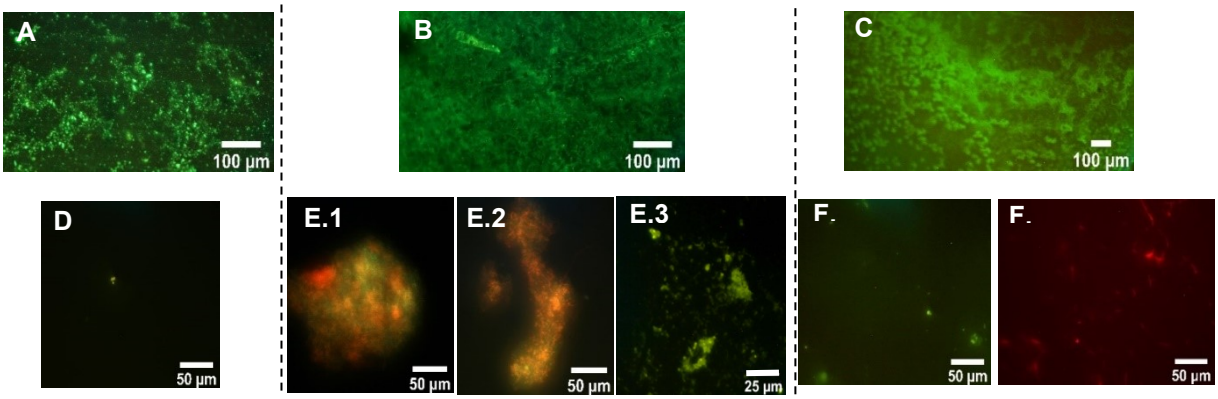

Figure 5.7. BacLight stained planktonic cell yield from CEMS cultivated biofilms. A-C t = 66 h (upon removal of initial treatment), D-F t = 234 h (after recovery from 456:24 µg/ml SMX:TMP exposure). A and D – biofilm (i), B and E – biofilm (ii), C and F- biofilm (iii). Live cells are represented by green fluorescence and dead cells are represented by red fluorescence.

In the biofilm biomass dislodged from the silicone tubing, no live cells were evident in any of the treatments at 99 h, at the first steady state reached after the initial treatments had been removed (Figure 5.8 A, D and G). Biomass removed after all lines had been exposed to 456:24 µg/ml SMX:TMP appeared to have more green cells (live) than red (dead) for biofilm (iii) (Figure 5.8 H), despite this biofilm not having detectable CO_2 production, and thus, metabolic activity (Figure 5.8 F), while biofilm (ii) appeared to have more red cells than green (Figure 5.8 E), and the control had similar proportions of red and green cells (Figure 5.8 B). Upon termination of the experiment (after the 608:32 µg/ml SMX:TMP spike), the biomass for all treatments were dominated by dead cells as expected based on the CO_2 profiles in Figure 5.3 D - F. The morphology of the cells at this time point differed from the previous biofilms, with biofilm (ii) having strings of biomass evident in some sections that were visualised (Figure 5.8 F1). Biofilm (iii) had very few visible cells upon termination of the experiment (Figure 5.8 I).

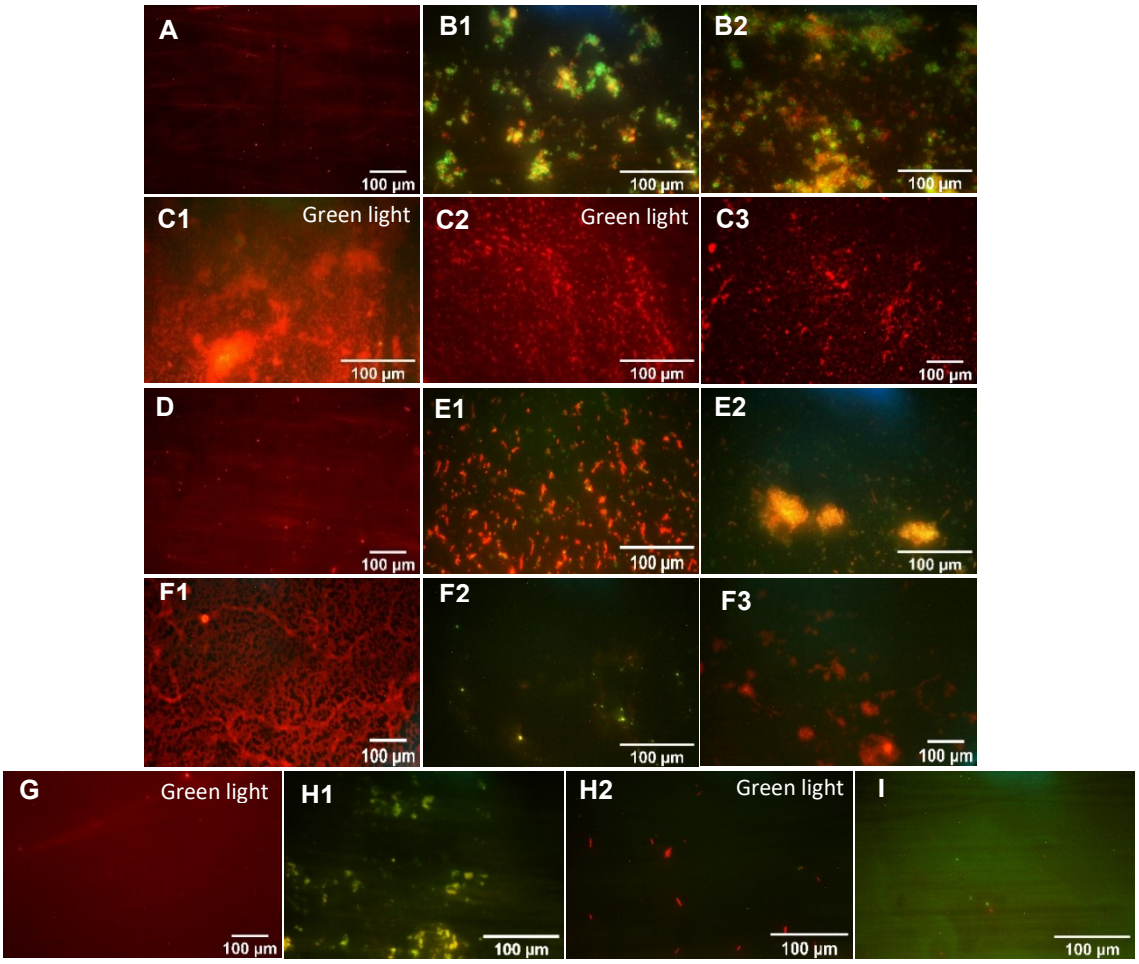

Figure 5.8. BacLight stained biofilm cultivated in CEMS. A-C – biofilm (i), D-F – biofilm (ii), G-I – biofilm (iii). A, D and G – biofilm at 99h (first steady state), B, E and H – biofilm at 190h (after 456:24 µg/ml SMX:TMP treatment), C, F, and I – biofilm at 285h (after 608:32 µg/ml SMX:TMP treatment).

5.4. Discussion

The metabolic profiles of biofilms showed different responses to each treatment. When inoculated and established in the absence of the two antimicrobials used in this study (biofilm (i)), the biofilms had a short lag phase before their metabolic activity increased exponentially. The metabolic activity of the first experiment overshot steady state, after which it decreased to values similar to the peak of the exponential phase. On the other hand, biofilms in the repeat experiment continued a gradual increase in metabolic rate (CO_2 production) until exposed to 456:24 µg/ml SMX:TMP (Figure 5.3 D). In conventional chemostat culture, the dilution rate with fresh medium is set below the specific growth rate of the culture, which prevents cell-washout (Kohanim et al., 2018). However, in natural streams and biofilm reactors used in biotechnology, flow (dilution or displacement) rates can exceed microbial growth rates without

loss of the culture or community due to biofilm persistence on available surfaces. In addition to preventing culture washout, the biofilms also serve as a 'cell nursery', as described by Bester et al. (2009), with biofilms yielding $\geq 10^8$ cells/cm^2 of attachment area per hour at dilution rates as high as 700 times the maximum growth rate of the culture. The dilution rate used in this study was 4.04/h in the CEMS portion of the reactor (Figure 5.1 B), which greatly exceeded the specific growth rates that can be expected by any of the biofilm community members, or overall growth rate of the community. With the CEMS reactors having a residence time of 15 minutes, planktonic cell counts thus provide a near-real time indication of biofilm-to-planktonic cell yield and thus comparable with the real-time measurement of CO_2 production as an indication of metabolic activity. Interestingly, biofilms (ii) and (iii) had no apparent metabolic activity and few viable planktonic cells were enumerated in biofilm (iii) (Figure 5.3 A and D). However, planktonic cell counts for biofilm (ii) were evident at $\pm 10^3$ CFU/ml, albeit low, prior to 72 h (Figure 5.3 B and E) and in the second repeat for biofilm (iii) (Figure 5.3 F). Performing carbon balances for biofilm growth over 408 hours, Bester at al. (2011) calculated that biofilms only utilise approximately 1% of the carbon input on planktonic cell yield. Thus, with evidence of some planktonic cell production, before 72 h, it can be inferred that metabolism occurred in biofilms (ii) and (iii), the CO_2 production was merely lower than the threshold of detection of the system. As such, these results show the role of biofilms in microbial proliferation, as opposed to the typical view that their primary function is survival. In addition, the microscopy images (Figure 5.7 A - C) show that live planktonic cells were evident in all treatments at 66 h (upon the removal of the initial MIC_{50} SMX:TMP and $ZnCl_2$ treatments in biofilms (i) and (ii)). This indicates that live cells were produced by the biofilms between 0-66 h from inoculation, despite the extremely low metabolic activity as indicated by the lack of measurable CO_2 production (Figure 5.3 B, C, E and F). The difference in the appearance between biofilms (i) and biofilm (ii) and (iii) (Figure 5.7 A - C) is an indication that the antimicrobial treatments potentially had some effect on the biofilm structure and EPS abundance, also indicated by the cloudy appearance in the planktonic cells from biofilms (ii) and (iii) (Figure 5.7 B and C).

The CO_2 profiles for the biofilms (i) and (ii) showed resemblance to a biphasic killing curve (exponential decrease in metabolic activity followed by a more gradual decrease) when exposed to 456:24 µg/ml SMX:TMP at 162 h, which is representative of antibiotic persistence (Balaban et al., 2019). Antibiotic persistent cells are not genetically resistant to antibiotics as they do not grow in the presence of antibiotics, however they are still able to survive –

potentially with more success in biofilms, with little to no metabolic activity which is often typical of the core zones of biofilms. Typically, once antimicrobial pressure is removed, the cells begin metabolising and the progeny respond in the same manner as a susceptible control with similar population sizes and similar susceptibilities to the antibiotic upon repeated exposure (Balaban et al., 2019). This is evident in Figure 5.3 where metabolism in biofilms (ii) and (iii) increased after the initial MIC_{50} antimicrobial treatment was removed after 66h. CO_2 production in treated biofilms reached similar steady state levels to biofilm (i), and biofilm (ii) responded in a similar manner to biofilm (i) when exposed to the 456:24 µg/ml SMX:TMP spike, while biofilm (iii) stopped metabolic activity immediately when spiked with 456:24 µg/ml SMX:TMP (Figure 5.3 C and F). Reversion back to a metabolic state was achieved after the 456:24 µg/ml antibiotic treatment was removed (186 h), suggesting that SMX:TMP exposure had little effect on the cells in the biofilms.

Despite the biofilms being grown in identical conditions, and similar CO_2 profiles and planktonic viable cell yield for the most part between replicates and different treatments, the biofilms in the second experiment showed different trends in MICs from the first (Figure 5.4). Kroukamp et al. (2010) highlighted that small changes in the inoculum can have a notable impact on biofilm development and function. Thus, slight variation in the RAS culture inoculum density or species composition between the repeated experiments, as well as the expected deviation from predictability when using a complex mixed culture opposed to a pure lab strain, may influence, and underestimate the MICs observed.

Initial exposure to MIC_{50} concentrations of SMX:TMP and $ZnCl_2$ in biofilms (ii) and (iii) respectively, did not affect the MICs of planktonic cells in reactor effluent when compared to biofilm (i) after exposure to 456:24 µg/ml SMX:TMP. Planktonic cells originating from all biofilms in the first experiment had increased MICs to SMX:TMP and decreased MICs to $ZnCl_2$ when exposed to the higher SMX:TMP concentrations. As there was no distinct difference in MIC trend between the different biofilms, it was initially concluded that exposure to sub-MIC concentrations of antibiotics or heavy metals did not induce a more resistant microbial community. Such observation between $ZnCl_2$ and antibiotic resistance has been reported by Ghazisaeedi et al., 2020 and Johanns et al., 2019. However, upon termination of the first experiment, the biofilm MICs for both SMX:TMP and $ZnCl_2$ were higher in biofilms (ii) and (iii) compared to biofilm (i). This suggests that biofilms initially treated with antimicrobials were more resistant to 608:32 µg/ml SMX:TMP than the biofilm (i) upon

termination of experiment 1 (Figure 5.4 A - C) which is similar to observations reported by Balaban et al. (2019).

Community analyses were subsequently performed to gain more insight into the response of these heterogeneous microbial communities after exposure to antimicrobials. The beta diversity NMDS plot community profiles in Figure 5.6 indicate that the biofilm communities for biofilm (i) and (ii) that were established in response to the 456:24 µg/ml SMX:TMP spike, were distinctly different from communities established in the first steady state. Communities at these time points in biofilm (iii) were more similar. Due to the lower exponential Shannon and inverse Simpson indices, it is evident that initial MIC_{50} SMX:TMP and $ZnCl_2$ treatment (biofilms (ii) and (iii)) selected communities that favoured dominant species, compared to biofilm (i) which had a more diverse community (Figure 5.5). This agreed with observations by others, that different types of treatment would select for specific community members with resistance mechanisms that allow persistence, resistance, or tolerance (Martínez, 2017). Here, it was hypothesised that species that readily form persister cells may have a strong influence on community composition and biofilm kinetics once the pressure of MIC_{50} treatments were removed (Figure 5.3). After the 456:24 µg/ml antibiotic spike to all lines, the control community was seen to further diversify (Figure 5.5). This was also found by Ye et al., 2020, however is contradictory to the observation made above by Martínez, 2017, who suggested that exposure of a bacterial community to high concentrations of antibiotics resulted in a more dominant community emerging, while exposure to a lower concentration diversifies the community. Although the community shifted between various stages of the experiment (Figure 5.6), the diversity of the communities in the MIC_{50} treated biofilms remained relatively similar (Figure 5.5). It has been suggested that persistent cells can be spontaneously derived from a steady state community (Balaban et al., 2019) and as a result, different species may be responsible for the decreased metabolic activity in biofilms (ii) and (iii) between 162 and 190h, conferring the shift in community composition that was observed at these time points (Figure 5.6). Overall, these results provide evidence for the extensive degree of metabolic redundancy amongst microbial communities that ensures maintenance of community function in the context of continuous fluctuation in the physical-chemical conditions in their environment.

A constant fluctuation of CO_2 production in biofilm (iii) was observed when antimicrobial pressure was removed (Figure 5.3 C and F). It was hypothesised that continuous sloughing events took place whereby a large portion of the biofilm biomass detached, or as mentioned previously, planktonic cells were produced as a means of biofilm proliferation and entered the

effluent. This is evident in Figure 5.3 where viable planktonic cells were higher when the CO_2 profile was decreasing (10^{12} CFU/ml at 86 h in the first repeat, and 10^4 CFU/ml at 118 h in the second repeat) and in the microscopy images (Figure 5.7C). Sloughing could arise from the potential of MIC_{50} $ZnCl_2$ exposure selecting for a microbial community dominated by species that are 'weak' biofilm formers that easily detach from the biofilm matrix, or a 'fight or flight' strategy to make resources available for resistant strains. Figure 5.8 supports this hypothesis by the relative quantity of live and dead cells observed in the biofilms when the first steady state was reached. Desai et al. (2019) indicated that *K. pneumoniae* strains that were weak biofilms formers tended to have a higher live-to-dead cell ratio compared to strains that were 'strong' biofilm formers, which have the opposite trend. According to Desai et al. (2019), biofilm (ii) showed resemblance to a biofilm consisting of a microbial community of strong biofilm formers as there were clearly higher numbers of red (dead) cells in the biofilm at the first steady state compared to live cells (Figure 5.8 E1 and E2), while biofilm (i) had similar dominance of live and dead cells (Figure 5.8 B1 and B2). This phenomenon was reinforced as biofilms (i) and (ii) had stable CO_2 profiles at steady state compared to biofilm (iii) (Figure 5.3). Figure 5.8 H1 (biofilm (iii)) shows live cells exclusively. To visualise the PI-stained cells (Figure 5.8 H2), the green filter (550 nm emission) was used due to a weak signal when blue light (480 nm emission) was used. It is evident that there were more live cells at this stage in the biofilm (iii). This, together with the fluctuating CO_2 steady state in biofilm (iii) (Figure 5.3C and F) suggests that the dominant species (indicated by the alpha-diversity in Figure 5.5) that result after initial MIC_{50} exposure to $ZnCl_2$ were unable to form biofilms with strong adherence. The potential bias of viability staining is recognised, and it is acknowledged that an absolute live or dead condition cannot be deduced from these stains; but rather utilize their value as an indication of viability to compare growth conditions.

Sub-MIC is defined as an antibiotic concentration that allows growth of a susceptible bacterial population, even if its growth rate is reduced (Andersson and Hughes, 2014). This could result in sub-MIC being any concentration below the MIC value. For example, 0.001 × MIC and 0.99 × MIC could both be considered sub-MIC. Biofilm form-function interactions are complex. As pure cultures primarily depend on differential gene expression to overcome a selective pressure, it was assumed that pure culture biofilms would have given a different scenario to what was determined in mixed community susceptibility testing; and more so, suspended cells in batch culture. A lower sub-MIC or MIC_{50} value may have been identified and perhaps different conclusions regarding the effect of MIC_{50} SMX:TMP and $ZnCl_2$ exposure on

antibiotic resistance would have been made. However, pure culture lab strains studies do not represent an environmental or clinical setting, and therefore, a mixed culture that may have species intrinsically resistant to antibiotics or metals, was used to determine sub-MIC in the context of this study. In addition to differential gene expression, these biofilm communities also have the option to shift community composition by increasing in the number of species that can better tolerate the change in environmental pressure, as well as biofilm structure though varying density, topography, and EPS composition. However, it was interesting to note that the Shannon and Simpson diversity indices were similar between biofilm and planktonic communities. This does not indicate that species composition was similar between the two phases, but rather that the community structure in terms of diversity and evenness was similar.

The mixed species biofilms' metabolic profiles observed in Figure 5.3 are of interest if applications to real-life situations are considered. Zinc has been found to limit growth of bacteria in the gastrointestinal tract (GIT) of pigs (Heo et al., 2013). A similar observation was seen in biofilm (iii) CO_2 profiles in Figure 5.3 where no biofilm metabolism was observed while 51, 40 and 20 µg/ml $ZnCl_2$ was supplemented in the TSB media. However, when the supplement was removed, biofilm metabolism rapidly increased, and an abundance of live biomass was evident in the planktonic cell yield (Figure 5.7 C). CO_2 declined abruptly at 120 h when $ZnCl_2$ was added to the growth medium again, however when sacrificing the biofilm at 186 h, live cells were observed (Figure 5.8 H1) despite metabolic activity below the detection limit. With $ZnCl_2$, and ZnO being supplemented to pig feed in high concentrations for growth promotion and prevention of diarrhoea in weaned piglets (Ghazisaeedi et al., 2020; Johanns et al., 2019; Zhang et al., 2020), bacterial growth in pig GITs may be inhibited due to constant exposure to the high concentrations of the supplement. In addition, these supplements could promote certain species in the GIT community to dominate, as observed in Figure 5.5. In this study, this community structure persisted, even after $ZnCl_2$ pressure had been removed. Thus, in an agricultural setting, dominant microbes in the GIT communities that are persistent against antimicrobials, can enter the environment through excretion and in the absence of high ZnO or $ZnCl_2$, have the potential to proliferate (as observed in the CO_2 profiles in Figure 5.3). A similar thought could be applied to humans taking SMX:TMP for treatment or prophylactic purposes and potentially the same could be applied to other antibiotics. The CO_2 metabolic profiles presented here raise a concern for the development of pathogenic biofilms if treatment is discontinued as the microscopy data (Figure 5.8 E) implies species present in biofilm (ii) were strong biofilm formers. In addition, the beta-diversity of the ARISA results (Figure 5.6) showed

community shifts in each biofilm after the respective treatments and concentrations. Applying this to a practical setting, although exposure to antibiotics and/or heavy metals did not result in increased resistance in mixed culture biofilms, sub-MIC exposure has the potential to alter the microbial communities of the gut microbiome or the environment and lead to development of persistent bacterial infections.

5.5. Conclusion

It was found that initial exposure of biofilms to sub-MIC concentrations of $ZnCl_2$ and SMX:TMP did not result in a marked increased resistance to SMX:TMP or $ZnCl_2$ in planktonic cells or biofilms compared to the initially untreated biofilms. However, exposure to these treatments appeared to result in the presence of persister cells, and metal and antibiotic tolerant cells that may have the potential to develop biofilms or proliferate if treatment was removed. This raises the concern that such response may facilitate persistent infections, and the disruption of the commensal bacterial community in humans, animals, or the environment, and proliferate when discharged into areas without antimicrobial pressure.

5.6. Acknowledgements

This work is based on the research supported in part by the National Research Foundation of South Africa (Grant numbers: 118159 and 130527)

APPENDIX C

SUPPLEMENTARY MATERIAL FOR CHAPTER 5

Sub-minimum inhibitory concentrations of zinc chloride and sulfamethoxazole-trimethoprim promote persister cell development in biofilms

C.1. Calculations for CO_2 conversion to µmol/hr.

C.1.1 Ideal gas equation:

$$V = \frac{nRT}{p}$$

Where *V*= molar volume (L), *n*= number of mols, *R*= universal gas constant (8.314 J/mol/K), *T*= temperature inside CO_2 analyser (K) and *p*= pressure inside the CO_2 analyser (pascals).

C.1.2 Molar flow rate (µmol/hr):

$$\frac{CO2 \times g}{V}$$

Where CO_2 is the concentration of CO_2 detected by the analyser (ppm), *g*= the gas flow rate of the ambient air pumped through the reactor (0.09 L/hr) and *V*= the molar volume.

C.2. Dilution rate calculation:

$$D = \frac{F}{V}$$

Where *D* is the dilution rate expressed per h, *F* is the measured flow rate of media through bioreactor = 13 ml/h, and *V* is the volume of the portion of the bioreactor that cultivates the biofilm.

Volume is calculated by the formula: $V=\pi r^2 \times h$,

where *r* is the silicone tubing internal radius in cm, and *h* is the length of the silicone tubing surrounded by Tygon tubing in cm.

$V= \pi(0.08)^2 \times 160$

$= 3.22\ cm^3 \approx ml$

Thus $D = \frac{13\ ml/h}{3.22\ ml}$

$= 4.04\ /h$

C.3. Retention time in biofilm portion of the bioreactor:

$$RT = \frac{V}{F}$$

$$= \frac{3.22\ ml}{13\ ml/h}$$

= 0.25 h = 15 min

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Ensuring water security for a rapidly increasing population is a necessity however, in low-middle income countries (LMIC) such as South Africa, this proves to be a challenge due to extensive contamination of freshwater resources, as well as insufficient and aging water treatment infrastructure (Herbig, 2019). Water reclamation is a strategy to ensure that water supply meets the current, and future demand however, safety of reclaimed water is often of concern. One of the factors that can impact safety is the presence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in these water resources that are vectors for the transmission of antimicrobial resistance (AMR) in the environment (Renew et al., 2001; Schwartz et al., 2003; Xu et al., 2015). To combat issues that arise due to AMR, it is important to acknowledge the role of multiple environments in the chain of dissemination and emergence. As a result of the "One Health" approach, that has been adopted globally for many health issues, being highly complex and multifaceted, understanding the role of the environment and the fate of AMR in this setting continues to be a challenge. Ideally, to truly understand the mechanisms and fate of AMR, interdisciplinary studies observing the link between AMR in animals (wild, domestic and livestock), the environment including water (rivers, lakes, dams, ground water etc.), sediment, soil, plants, and other natural environments, and humans (domestic, industrial, and clinical settings) are needed. Realistically however, studies on this scale are near impossible due to resource-, funding-, personnel-, and time constraints. Many studies addressing environmental AMR focus on WWTW and surface waters. While this contributes to what is known about AMR, results are often particular to a certain niche in terms of the selected study site, the selected target antibiotics, and the country in which the study took place, which may influence the outcome. Data on AMR in the environment is limited in SA and as a result, the spatio-temporal study presented here should add value through the focus on the prominent areas that may contribute to AMR in society, in addition to the role of WWTW in AMR dissemination.

Culture methods performed in Chapter 3 reinforced the complex nature of environmental AMR studies. This study confirmed that ARB and ARG were present in the treated effluent and have potential to persist in the environment if repurposed for irrigation, drinking water for cattle, and industrial processes. However, in contrast to most studies suggesting that WWTW are culprits in AMR dissemination (Guo et al., 2017; Hultman et al., 2018; Rizzo et al., 2013), it was found that the number of bacteria resistant to amoxicillin, sulfamethoxazole, gentamicin

and colistin in two WWTW in Cape Town, decreased in the effluent compared to the influent. *Bla*$_{KPC-1}$ was the only target gene that increased in copies/16S rDNA in the effluent compared to the influent, while *mcr3, sul1* and *sul2* gene copies were reduced in the effluent by 69 -100 %. It was observed that a plug flow effluent treatment system reduced ARB and ARG more efficiently compared to a mixed flow effluent treatment, and it was suggested that through the implementation of appropriate operation, WWTW may play a seminal role in reducing the dissemination of AMR in the environment rather than contributing to it. Exposing environmental microbial communities to antibiotics have the potential to select for species that are intrinsically resistant to antibiotics as seen in the data obtained. However, it is acknowledged that different results might be observed in different locations, different wastewater treatment strategies and if different antibiotics were tested. The findings of this Chapter could assist in determining more effective treatment strategies for AMR removal that could be implemented in WWTW upgrades or modification.

From Chapter 3 it is evident that AMR is more prominent in the influent of WWTW compared to the effluent. As a result, the focus shifted to the broader community to determine where ARB in the influent might originate or where potential environmental AMR hotspots might be. Chapter 4 discussed a spatio-temporal study in Stellenbosch. Here it was confirmed that surface waters close to anthropogenic activities (informal settlements, domestic, and industrial environments) had higher percentages of resistant bacteria, particularly towards sulfamethoxazole and gentamicin, antibiotics frequently used in healthcare settings. Surface waters in close proximity to agricultural settings had higher percentages of bacteria resistant to carbapenems and colistin compared to bacteria resistant to sulfamethoxazole and gentamicin. Despite biofilms being known to be more resistant to antibiotics, it was found that aqueous samples had a higher percentage of resistant bacteria. Various factors may contribute to this result, such as variations in stream flow that resulted in sloughing and erosion of peripheral cells of the biofilm that tend to be more active in HGT of ARG, as well as biofilms acting as cell nurseries through continuous yield of resistant bacteria to the bulk aqueous phase. In addition, higher levels of resistant bacteria were found in the sediment samples compared to biofilm samples. The solid particulate matter of sediment has a larger surface area compared to larger surfaces, which increases the surface area available for biofilm attachment and increased presence of resistant bacteria. Future studies should consider conducting environmental biofilm studies without disrupting the biofilm by using devices physically

placed at the sample site to allow biofilms to attach and mature, that can be removed when needed for analysis.

The WWTW described in Chapter 4 showed a reduction in ARB in the effluent, reinforcing the results observed Chapter 3. Monthly variation in resistance profiles were evident at all sites, again emphasising the complex nature of environmental studies whereby unique and isolated events may have a strong influence on results. One of the notable variations in the monthly results was the increased percentage of ARB that were present in the wastewater effluent when scheduled power cuts (distributed load shedding lasting 2 hours-plus at a time to prevent largescale collapse of the power grid), was implemented. This serves as a good example of the growing lack of infrastructure in LMIC, which raises the concern for new health risks that may emerge (Herbig, 2019). While the WWTW in this study appeared to be compliant to effluent standards, lapses in functioning as observed in load shedding months with higher ARB in WWTW effluent impacts public health, and by extension the public's confidence, which shows that focussing primarily on WWTW as hotspots of AMR is not enough, and should incorporate broader societal challenges. One such example is the over-reliance on disinfectants in high-income / developed countries. Ironically, this may promote an environment primed for AMR proliferation by removing commensal bacteria that would otherwise assist in reducing ARB by competitive exclusion (Stone et al., 2020).

Metagenomics has become a useful tool with recent developments making it more cost effective (Escobar-Zepeda et al., 2015). As more information can be obtained from a sample by using a metagenomic approach compared to culturing methods, it has been widely used in environmental studies as a more representative construct of AMR in the environment (Guo et al., 2017; Kneis et al., 2019; Yang et al., 2014). As a result, a metagenomic aspect of this study (Chapter 4) was included. The data suggests that efflux pumps might have a greater role to play in AMR than specific plasmid mediated resistance genes for particular antibiotics due to the abundance of genes encoding efflux pumps compared to target ARG despite antibiotic exposure. This was also evident in the carbapenem resistant single colonies that were isolated from culture media. It was found that the identified species in the culture-based method were also the dominant species detected by metagenome analysis and the dominant resistance genes detected were similar across both methods, suggesting that even with culture bias, plating techniques yield results that were representative of the sample as a whole. This study focused on the antibiotic resistance genes specific for the antibiotics selected for this study, however the amount of information that can be drawn from the metagenomic analysis far exceeds what

is presented in this dissertation based on what the specific objectives were. This proves to be a challenge with other metagenomic studies as well that generate a plethora of information yet come to similar conclusions to what is presented here in Chapter 3 and 4 by culture-based approaches (Tang et al., 2016). Although generating data of this nature is useful in monitoring studies and determining new resistance genes and changes in microbial communities (De, 2019; Tang et al., 2016), global databases need to be accessed, and collaboration needs to occur (Duarte et al., 2020) if transfer of ARG and persistence of ARB in the environment is to be understood and real solutions to combating AMR are identified.

During the sampling campaigns in the study conducted in Chapter 4, it was evident that other contaminants that are present in the environment contribute to the pollution and poor health of surface waters. Amongst these are heavy metals which are naturally present in the environment and are relevant to a number of applications. Microbial bioremediation has been implemented to reduce the concentration of these to below toxic levels. As a result, the likelihood of organisms that are exposed to heavy metals being resistant to antibiotics has been questioned (Alam and Imran, 2014; Turner, 2017). Of particular interest in Chapter 5 was $ZnCl_2$ due to its use in many products and applications, as well as in animal husbandry as a prophylactic treatment and growth promoter (Johanns et al., 2019). Using a carbon-dioxide evolution measurement system (CEMS), it was determined that resistance to SMX:TMP per se did not increase in mixed-community biofilms exposed to sub-inhibitory concentrations of SMX:TMP and $ZnCl_2$. However, it appeared that the biofilms exposed to these contaminants established mechanisms of persistence and tolerance as biofilm metabolism returned to normal levels when the various treatments were removed. Communities exposed to these treatments resulted in different beta diversities at various concentrations. Biofilms not exposed to any initial treatment showed increased alpha diversity and evenness when exposed to SMX:TMP, while biofilms initially exposed to sub-inhibitory SMX:TMP concentrations had similar alpha diversity and evenness regardless of the subsequent treatment concentrations. It was also suggested that mixed community biofilm initially exposed to $ZnCl_2$ consisted of dominant species that were weak biofilm formers that frequently slough off peripheral cells, in addition to production of new cells that enter the planktonic phase to promote dispersal. Due to the frequent use of heavy metals and antibiotics in industry, humans, and being added to animal feed, findings of this experiment have relevance to real-life scenarios where exposure to these compounds may not result in AMR but development of antibiotic or heavy-metal tolerant cells the enter the environment, which may pose an equally problematic risk for public health.

Clearly, multiple factors influence AMR dissemination, with the ability of bacteria to adapt to their environment posing a notable challenge to efforts aimed at controlling such spread; and will continue to be a concern even if alternative treatment strategies are developed in the future. The findings presented in this dissertation emphasise the need to support a proactive approach to address the AMR crisis by limiting the entry of ARB, antibiotics and other contaminants into the environment, and especially freshwater resources.

CHAPTER 7

REFERENCES

Aarestrup, F. M., Hasman, H., Jensen, L. B., Moreno, M., Herrero, I. A., Domínguez, L., Finn, M., and Franklin, A. (2002). Antimicrobial resistance among *Enterococci* from pigs in three European countries. *Applied and Environmental Microbiology*, 68(8), 4127–4129. https://doi.org/10.1128/AEM.68.8.4127-4129.2002

Abbott, S. L. (2011). *Klebsiella, Enterobacter, Citrobacter, Serratia, Plesiomonas*, and other Enterobacteriaceae. In Manual of Clinical Microbiology, 10th Edition, 639–657. *American Society of Microbiology.*

Adefisoye, M. A., and Okoh, A. I. (2016). Identification and antimicrobial resistance prevalence of pathogenic *Escherichia coli* strains from treated wastewater effluents in Eastern Cape, South Africa. *MicrobiologyOpen*, 5(1), 143–151. https://doi.org/10.1002/mbo3.319

Adegoke, A. A., Amoah, I. D., Stenström, T. A., Verbyla, M. E., and Mihelcic, J. R. (2018). Epidemiological evidence and health risks associated with agricultural reuse of partially treated and untreated wastewater: A review. *Frontiers in Public Health*, 6(December). https://doi.org/10.3389/fpubh.2018.00337

Alam, M., and Imran, M. (2014). Multiple antibiotic resistances in metal tolerant *E. coli* from hospital waste water. *Bioinformation*, 10(5), 267–272. https://doi.org/10.6026/97320630010267

Alcalde-Rico, M., Hernando-Amado, S., Blanco, P., and Martínez, J. L. (2016). Multidrug efflux pumps at the crossroad between antibiotic resistance and bacterial virulence. *Frontiers in Microbiology*, 7(1483). https://doi.org/10.3389/fmicb.2016.01483

Alcock, J. (2018). Evolutionary emergency medicine. *Evolution Medicine*. https://evolutionmedicine.com/2018/02/05/evolutionary-emergency-medicine/

Andersson, D. I., and Hughes, D. (2014). Microbiological effects of sublethal levels of antibiotics. *Nature Reviews Microbiology*, 12(7), 465–478.

Alexander, J., Hembach, N., & Schwartz, T. (2020). Evaluation of antibiotic resistance dissemination by wastewater treatment plant effluents with different catchment areas in Germany. Scientific Reports, 10(1), 1–9. https://doi.org/10.1038/s41598-020-65635-4

Andrews, J. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*, 48, 5-16. https://doi.org/10.1093/jac/48.suppl_1.5

Archer, E., Petrie, B., Kasprzyk-Hordern, B., and Wolfaardt, G. M. (2017). The fate of pharmaceuticals and personal care products (PPCPs), endocrine disrupting contaminants (EDCs), metabolites and illicit drugs in a WWTW and environmental waters. *Chemosphere*, 174, 437–446. https://doi.org/10.1016/j.chemosphere.2017.01.101

Ash, R. J., Mauck, B., and Morgan, M. (2002). Antibiotic resistance of gram-negative bacteria in rivers, United States. *Emerging infectious diseases*, 8(7), 713–716. https://doi.org/10.3201/eid0807.010264

Audi, H., Knox, J., Hall, W., McDonnell, A., Seshadri, A., Mudd, J., Truscott-Reid, N., Macdonald, O., Toxvaerd, F., and Woodford, N. (2016). Tackling drug-resistant infections globally: Final report and recommendations. Available at: https://amr-review.org/Publications.html

Baker-Austin, C., Wright, M. S., Stepanauskas, R., and McArthur, J. V. (2006). Co-selection of antibiotic and metal resistance. *Trends in Microbiology*, 14(4), 176–182. https://doi.org/https://doi.org/10.1016/j.tim.2006.02.006

Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., Brynildsen, M. P., Bumann, D., Camilli, A., Collins, J. J., Dehio, C., Fortune, S., Ghigo, J. M., Hardt, W. D., Harms, A., Heinemann, M., Hung, D. T., Jenal, U., Levin, B. R., Michiels, J., Storz, G., Tan, M. W.,Tenson, T., Van Melderen, L., and Zinkernagel, A. (2019). Definitions and guidelines for research on antibiotic persistence. *Nature Reviews Microbiology*, 17(7), 441–448. https://doi.org/10.1038/s41579-019-0196-3

Balcázar, J. L., Subirats, J., and Borrego, C. M. (2015). The role of biofilms as environmental reservoirs of antibiotic resistance. *Frontiers in Microbiology*, 6(October), 1216. https://doi.org/10.3389/fmicb.2015.01216

Band, V.I., Hufnagel, D.A., Jaggavarapu, S., Sherman, E.X., Wozniak, J.E., Satola, S. W., Farley, M.M., Jacob, J. T., Burd E. M. & Weiss, D. M. (2019). Antibiotic combinations that exploit heteroresistance to multiple drugs effectively control infection. *Nature Microbiology*, 4, 1627–1635. https://doi.org/10.1038/s41564-019-0480-z

Barber, M., and Rozwadowska-Dowzenko, M. (1948). Infection by penicillin-resistant staphylococci. *Lancet*, 2(6530), 641–644. https://doi.org/10.1016/s0140-6736(48)92166-7

Bassetti, M., Vena, A., Croxatto, A., Righi, E., and Guery, B. (2018). How to manage *Pseudomonas aeruginosa* infections. *Drugs in Context*, 7, 212527. https://doi.org/10.7573/dic.212527

Batt, A. L., Bruce, I. B., and Aga, D. S. (2006). Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. *Environmental Pollution*, 142(2), 295–302.

Baynes, R. E., Dedonder, K., Kissell, L., Mzyk, D., Marmulak, T., Smith, G., Tell, L., Gehring, R., Davis, J., and Riviere, J. E. (2016). Health concerns and management of select veterinary drug residues. *Food and Chemical Toxicology*, 88, 112–122. https://doi.org/10.1016/j.fct.2015.12.020

Bazzi, W., Abou Fayad, A. G., Nasser, A., Haraoui, L. P., Dewachi, O., Abou-Sitta, G., Nguyen, V. K., Abara, A., Karah, N., Landecker, H., Knapp, C., McEvoy, M. M., Zaman, M. H., Higgins, P. G., and Matar, G. M. (2020). Heavy metal toxicity in armed conflicts potentiates AMR in *A. baumannii* by selecting for antibiotic and heavy metal co-resistance mechanisms. *Frontiers in Microbiology*. 11, 68. https://doi.org/10.3389/fmicb.2020.00068

Bello-López, J. M., Cabrero-Martínez, O. A., Ibáñez-Cervantes, G., Hernández-Cortez, C., Pelcastre-Rodríguez, L. I., Gonzalez-Avila, L. U., and Castro-Escarpulli, G. (2019). Horizontal gene transfer and its association with antibiotic resistance in the genus *Aeromonas* spp. *Microorganisms.* 7(9). https://doi.org/10.3390/microorganisms7090363

Bengtsson-Palme, J., and Larsson, D. G. J. (2016). Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. *Environment International*, 86, 140–149. https://doi.org/10.1016/j.envint.2015.10.015

Benson, C. A., Brooks, J. T., Holmes, K. K., Kaplan, J. E., Masur, H., and Pau, A. (2009). Guidelines for prevention and treatment opportunistic infections in HIV-infected adults and adolescents; recommendations from CDC, the National Institutes of Health, and the HIV Medicine Association/Infectious Diseases Society of America. Available at: https://www.cdc.gov/mmwr/preview/mmwrhtml/rr58e324a1.htm

Berendonk, T. U., Manaia, C. M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Burgmann, H., Sorum, H., Norstrom, M., Pons, M.-N. N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J. L., Bürgmann, H., Sørum, H., Norström, M., Pons, M. N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F. and Martinez, J. L. (2015). Tackling antibiotic resistance: the environmental framework. *Nature Reviews Microbiology*, 13(5), 310. https://doi.org/10.1038/nrmicro3439

Berg, J., Thorsen, M. K., Holm, P. E., Jensen, J., Nybroe, O., and Brandt, K. K. (2010). Cu exposure under field conditions coselects for antibiotic resistance as determined by a novel cultivation-independent bacterial community tolerance assay. *Environmental Science and Technology*, 44(22), 8724–8728. https://doi.org/10.1021/es101798r

Bernhoft, A., Amundsen, C. E., Källqvist, T., Rudi, K., Sogn, T., Yazdankhah, S., and Ørnsrud, R. (2014). Zinc and copper in pig and poultry production – fate and effects in the food chain and the environment. Available at: https://vkm.no/download/18.2994e95b15cc54507161edc3/1501777318338/e06b487e66.pdf

Bester, E., Kroukamp, O., Hausner, M., Edwards, E. A., and Wolfaardt, G. M. (2011). Biofilm form and function: carbon availability affects biofilm architecture, metabolic activity and planktonic cell yield. *Journal of Applied Microbiology*, 110(2), 387–398. https://doi.org/10.1111/j.1365-2672.2010.04894.x

Bester, E., Edwards, E., and Wolfaardt, G. (2009). Planktonic cell yield is linked to biofilm development. *Canadian Journal of Microbiology*, 55(10), 1195–1206. https://doi.org/10.1139/W09-075

Bhasin, S., Shukla, A. N., and Shrivastava, S. (2015). Observation on *Pseudomonas aeruginosa* in Kshipra river with relation to anthropogenic activities. *International Journal of Current Microbiology and Applied Sciences,* 4(4). https://www.ijcmas.com/vol-4-4/Shivi%20Bhasin,%20et%20al.pdf

Bonomo, R. A. (2017). β-Lactamases: A focus on current challenges. *Perspectives in Medicine,* 7(1), a025239. https://doi.org/10.1101/cshperspect.a025239

Branswell, H. (2017). Nevada woman dies of superbug resistant to all available US antibiotics. *Stat News*. Available at: https://www.statnews.com/2017/01/12/nevada-woman-superbug-resistant/

Briffa, J., Sinagra, E., and Blundell, R. (2020). Heavy metal pollution in the environment and their toxicological effects on humans. *Heliyon*, 6(9), e04691. https://doi.org/10.1016/j.heliyon.2020.e04691

Bryers, J. D. (2008). Medical biofilms. *Biotechnology and Bioengineering,* 100(1), 1–18. https://doi.org/10.1002/bit.21838

Cai, Y., Chai, D., Wang, R., Liang, B., and Bai, N. (2012). Colistin resistance of *Acinetobacter baumannii*: clinical reports, mechanisms and antimicrobial strategies. *Journal of Antimicrobial Chemotherapy*, 67(7), 1607–1615. https://doi.org/10.1093/jac/dks084

Caicedo, C., Rosenwinkel, K. H., Exner, M., Verstraete, W., Suchenwirth, R., Hartemann, P., and Nogueira, R. (2019). *Legionella* occurrence in municipal and industrial wastewater treatment plants and risks of reclaimed wastewater reuse: Review. *Water Research*, 149, 21–34. https://doi.org/10.1016/j.watres.2018.10.080

Cantas, L., Shah, S. Q. A., Cavaco, L. M., Manaia, C. M., Walsh, F., Popowska, M., Garelick, H., Burgmann, H., Sorum, H., Bürgmann, H., Sørum, H., Burgmann, H., and Sorum, H. (2013). A brief multi-disciplinary review on antimicrobial resistance in medicine and its linkage to the global environmental microbiota. *Frontiers in Microbiology*, 4(May), 96. https://doi.org/10.3389/fmicb.2013.00096

Carroll, L. M., Gaballa, A., Guldimann, C., Sullivan, G., Henderson, L. O., and Wiedmann, M. (2019). Identification of novel mobilized colistin resistance gene mcr-9 in a multidrug-resistant, colistin-susceptible *Salmonella enterica* serotype *typhimurium* isolate. *MBio*, 10(3). https://doi.org/10.1128/mBio.00853-19

Cavaco, L. M., Hasman, H., Aarestrup, F. M., Wagenaa, J. A., Graveland, H., Veldman, K., Mevius, D., Fetsch, A., Tenhagen, B. A., Porrero, M. C., Dominguez, L., Granie, S. A., Jouy, E., Butaye, P., Kaszanyitzky, E., Dán, A., Zmudzki, J., Battisti, A., and Franco, A. (2011). Zinc resistance of *Staphylococcus aureus* of animal origin is strongly associated with methicillin resistance. *Veterinary Microbiology*, 150(3–4), 344–348. https://doi.org/10.1016/j.vetmic.2011.02.014

Centres for Disease Control and Prevention. (2013). Antibiotic resistance threats in the United States. Centres for Disease Control and Prevention, US Department of Health and Human Services. Available at: https://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.p

Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D., and Buret, A. (1999). The Calgary Biofilm Device: New technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *Journal of Clinical Microbiology*, 37(6), 1771–1776. https://doi.org/10.1128/jcm.37.6.1771-1776.1999

Chen, J., Li, J., Zhang, H., Shi, W., and Liu, Y. (2019). Bacterial heavy-metal and antibiotic resistance genes in a copper tailing dam area in northern China. *Frontiers in Microbiology*, 10. https://doi.org/10.3389/fmicb.2019.01916

Cheng, G., Ning, J., Ahmed, S., Huang, J., Ullah, R., An, B., Hao, H., Dai, M., Huang, L., Wang, X., and Yuan, Z. (2019). Selection and dissemination of antimicrobial resistance in Agri-food production. *Antimicrobial Resistance and Infection Control,* 8(1), 1–13). https://doi.org/10.1186/s13756-019-0623-2

City of Cape Town. (n.d.). Our shared water future. Available at: https://resource.capetown.gov.za/documentcentre/Documents/City strategies, plans and frameworks/Cape Town Water Strategy.pdf

Clinical and Laboratory Standards Institute. (2015). Performance Standards for antimicrobial susceptibility testing; twenty-fifth informational supplement. Available at: https://kaldur.landspitali.is/focal/gaedahandbaekur/gnhsykla.nsf/5e27f2e5a88c898e00256500003c98c2/9c4f4955ccb9f8100025751a0046b075/$FILE/ATTIGBN7.pdf/M100-S25%20Performance%20Standards%20for%20Antimicrobial%20Susceptibility%20Testing.pdf

Clinical and Laboratory Standards Institute. (2018). M100 Performance Standards for antimicrobial susceptibility testing. 28th edition. Available at: http://file.qums.ac.ir/repository/mmrc/CLSI-2018-M100-S28.pdf

Codjoe, F., and Donkor, E. (2017). Carbapenem resistance: A review. *Medical Sciences*, 6(1), 1. https://doi.org/10.3390/medsci6010001

Corcoran, M., Morris, D., De Lappe, N., O'Connor, J., Lalor, P., Dockery, P., and Cormican, M. (2014). Commonly used disinfectants fail to eradicate *Salmonella enterica* biofilms from food contact surface materials. *Applied and Environmental Microbiology,* 80(4), 1507-1514. https://doi.org/10.1128/AEM.03109-13

Crouse, J., and Amorese, D. (1987). Ethanol precipitation: ammonium acetate as an alternative to sodium acetate. *Focus*, 9(2), 3–5. https://doi.org/10.1007/BF02668762

Czekalski, N., Berthold, T., Caucci, S., Egli, A., and Bürgmann, H. (2012). Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. *Frontiers in Microbiology*, 3(March), 1–18. https://doi.org/10.3389/fmicb.2012.00106

Dafale, N. A., Srivastava, S., and Purohit, H. J. (2020). Zoonosis: An emerging link to antibiotic resistance under "One Health Approach." *Indian Journal of Microbiology*, 60(2), 139–152. https://doi.org/10.1007/s12088-020-00860-z

Das, S., Singh, S., Matchado, M. S., Srivastava, A., and Bajpai, A. (2019). Biofilms in human health. *Biofilms in Human Diseases: Treatment and Control*, 27–42. https://doi.org/10.1007/978-3-030-30757-8_3

De, R. (2019). Metagenomics: Aid to combat antimicrobial resistance in diarrhea. *Gut Pathogens*, 11(1), 47). *BioMed Central*. https://doi.org/10.1186/s13099-019-0331-8

Department of Water Affairs. (2013). Green Drop Report: Municipal and private wastewater systems. Available at: http://www.dwa.gov.za/Dir_WS/GDS/Docs/DocsDefault.aspx

Desai, S., Sanghrajka, K., and Gajjar, D. (2019). High adhesion and increased cell death contribute to strong biofilm formation in *Klebsiella pneumoniae*. *Pathogens*, 8(4). https://doi.org/10.3390/pathogens8040277

Dobias, J., Poirel, L., and Nordmann, P. (2017). Cross-resistance to human cationic antimicrobial peptides and to polymyxins mediated by the plasmid-encoded *MCR-1*? *Clinical Microbiology and Infection*, 23(9), 676.e1-676.e5. https://doi.org/https://doi.org/10.1016/j.cmi.2017.03.015

dos Santos, L. D. R., Furlan, J. P. R., Ramos, M. S., Gallo, I. F. L., de Freitas, L. V. P., and Stehling, E. G. (2020). Co-occurrence of *mcr-1*, *mcr-3*, *mcr-7* and clinically relevant antimicrobial resistance genes in environmental and fecal samples. *Archives of Microbiology*, 1, 1–6. https://doi.org/10.1007/s00203-020-01890-3

Du, H., Chen, L., Tang, Y.-W., and Kreiswirth, B. N. (2016). Emergence of the *mcr-1* colistin resistance gene in carbapenem-resistant Enterobacteriaceae. *The Lancet Infectious Diseases*, 16(3), 287–288.

Duarte, A. S. R., Stärk, K. D. C., Munk, P., Leekitcharoenphon, P., Bossers, A., Luiken, R., Sarrazin, S., Lukjancenko, O., Pamp, S. J., Bortolaia, V., Nissen, J. N., Kirstahler, P., Van Gompel, L., Poulsen, C. S., Kaas, R. S., Hellmér, M., Hansen, R. B., Gomez, V. M., and Hald, T. (2020). Addressing learning needs on the use of metagenomics in antimicrobial resistance surveillance. *Frontiers in Public Health*, 8, 38. https://doi.org/10.3389/fpubh.2020.00038

Duong, H. A., Pham, N. H., Nguyen, H. T., Hoang, T. T., Pham, H. V., Pham, V. C., Berg, M., Giger, W., and Alder, A. C. (2008). Occurrence, fate and antibiotic resistance of fluoroquinolone antibacterials in hospital wastewaters in Hanoi, Vietnam. *Chemosphere*, 72(6), 968–973. https://doi.org/10.1016/j.chemosphere.2008.03.009

Duran, N., Ozer, B., Duran, G. G., Onlen, Y., and Demir, C. (2012). Antibiotic resistance genes and susceptibility patterns in Staphylococci. *Indian Journal of Medical Research*, 135(3), 389–396. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3361877/

Ebomah, K. E., and Okoh, A. I. (2020). *Enterobacter cloacae* harbouring *bla NDM-1* , *bla KPC* , and *bla OXA-48*-like carbapenem-resistant genes isolated from different environmental sources in South Africa. *International Journal of Environmental Studies*, 1–14. https://doi.org/10.1080/00207233.2020.1778274

Edokpayi, J. N., Odiyo, J. O., and Durowoju, O. S. (2017). Impact of wastewater on surface water quality in developing countries: A case study of South Africa. *Water Quality*. https://doi.org/10.5772/66561

Eduardo-Correia, B., Morales-Filloy, H., and Abad, J. P. (2020). Bacteria From the Multi-Contaminated Tinto River Estuary (SW, Spain) Show High Multi-Resistance to Antibiotics and Point to Paenibacillus spp. as Antibiotic-Resistance-Dissemination Players. *Frontiers in Microbiology,* 10(January), 3071. https://doi.org/10.3389/fmicb.2019.03071

Ekwanzala, M. D., Dewar, J. B., Kamika, I., and Momba, M. N. B. (2018). Systematic review in South Africa reveals antibiotic resistance genes shared between clinical and environmental settings. *Infection and Drug Resistance*, 11, 1907–1920. https://doi.org/10.2147/IDR.S170715

El Garch, F., Sauget, M., Hocquet, D., LeChaudee, D., Woehrle, F., and Bertrand, X. (2017). *mcr-1* is borne by highly diverse *Escherichia coli* isolates since 2004 in food-producing animals in Europe. *Clinical Microbiology and Infection*, 23(1), 51 e1-51 e4. https://doi.org/10.1016/j.cmi.2016.08.033

Escobar-Zepeda, A., De León, A. V. P., and Sanchez-Flores, A. (2015). The road to metagenomics: From microbiology to DNA sequencing technologies and bioinformatics. *Frontiers in Genetics*, 6,(December), 348). https://doi.org/10.3389/fgene.2015.00348

Essack, S. Y., Hall, L. M. C., Pillay, D. G., McFadyen, M. L., and Livermore, D. M. (2001). Complexity and diversity of *Klebsiella pneumoniae* strains with extended-spectrum β-lactamases isolated in 1994 and 1996 at a teaching hospital in Durban, South Africa. *Antimicrobial Agents and Chemotherapy*, 45(1), 88–95.

European Committee on Antimicrobial Susceptibility Testing. (2010). Rationale for the EUCAST clinical breakpoints: version 1.0. Available at: https://www.eucast.org/ast_of_bacteria/previous_versions_of_documents/

European Committee on Antimicrobial Susceptibility Testing. (2010). Amoxicillin: Rationale for the clinical breakpoints. Available at: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Rationale_documents/Amoxicillin_rationale_Nov2010_v_1.0.pdf

European Committee on Antimicrobial Susceptibility Testing. (2017). Breakpoint tables for interpretation of MICs and zone diameters: version 7. Available at: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.1_Breakpoint_Tables.pdf

European Medicines Agency. (2016). Updated advice on the use of colistin products in animals within the European Union: development of resistance and possible impact on human and animal health. *Science Medicines Health*, 44(July), 56. Available at: internal-pdf://187.165.255.1/Updated advice on the use of colistin products.pdf

Felske, A., Akkermans, A. D., and De Vos, W. M. (1998). Quantification of 16S rDNAs in complex bacterial communities by multiple competitive reverse transcription-PCR in temperature gradient gel electrophoresis fingerprints. *Applied and Environmental Microbiology*, 64(11), 4581–4587. http://www.ncbi.nlm.nih.gov/pubmed/9797325

Féria, C., Ferreira, E., Correia, J. D., Gonçalves, J., and Caniça, M. (2002). Patterns and mechanisms of resistance to β-lactams and β-lactamase inhibitors in uropathogenic *Escherichia coli* isolated from dogs in Portugal. *Journal of Antimicrobial Chemotherapy*, 49(1), 77–85. https://doi.org/10.1093/jac/49.1.77

Ferreira Da Silva, M., Vaz-Moreira, I., Gonzalez-Pajuelo, M., Nunes, O. C., and Manaia, C. M. (2007). Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. *FEMS Microbiology Ecology*, 60(1), 166–176. https://doi.org/10.1111/j.1574-6941.2006.00268.x

Figueras Salvat, M. J., and Ashbolt, N. (2019). Aeromonas. In Global Water Pathogen Project. Michigan State University. https://doi.org/10.14321/waterpathogens.21

Fito, J., and Van Hulle, S. W. H. (2020). Wastewater reclamation and reuse potentials in agriculture: towards environmental sustainability. *Environment, Development and Sustainability,* 1–24. https://doi.org/10.1007/s10668-020-00732-y

Fouz, N., Pangesti, K. N. A., Yasir, M., Al-Malki, A. L., Azhar, E. I., Hill-Cawthorne, G. A., and El Ghany, M. A. (2020). The contribution of wastewater to the transmission of antimicrobial resistance in the environment: Implications of mass gathering settings. *Tropical Medicine and Infectious Disease*, 5(1). https://doi.org/10.3390/tropicalmed5010033

Friedrich, E., Pillay, S., and Buckley, C. A. (2009). Carbon footprint analysis for increasing water supply and sanitation in South Africa: a case study. *Journal of Cleaner Production*, 17(1), 1–12. https://doi.org/10.1016/j.jclepro.2008.03.004

Furlan, J. P. R., Dos Santos, L. D. R., Ramos, M. S., Gallo, I. F. L., Moretto, J. A. S., and Stehling, E. G. (2020). Occurrence of clinically relevant antimicrobial resistance genes, including *mcr-3* and *mcr-7.1* , in soil and water from a recreation club. *International Journal of Environmental Health Research*, 1–10. https://doi.org/10.1080/09603123.2020.1799953

Gales, A. C., Jones, R. N., and Sader, H. S. (2011). Contemporary activity of colistin and polymyxin B against a worldwide collection of Gram-negative pathogens: Results from the SENTRY antimicrobial surveillance program (2006-09). *Journal of Antimicrobial Chemotherapy*, 66(9), 2070–2074. https://doi.org/10.1093/jac/dkr239

Gao, R., Hu, Y., Li, Z., Sun, J., Wang, Q., Lin, J., Ye, H., Liu, F., Srinivas, S., Li, D., Zhu, B., Liu, Y. H., Tian, G. B., and Feng, Y. (2016). Dissemination and Mechanism for the *MCR-1* Colistin Resistance. *PloS Pathogens*, 12(11), e1005957. https://doi.org/10.1371/journal.ppat.1005957

Gelband, H., Molly Miller, P., Pant, S., Gandra, S., Levinson, J., Barter, D., White, A., and Laxminarayan, R. (2015). The state of the world's antibiotics 2015. 8(2), 30–34. Available at: https://cddep.org/wp-content/uploads/2017/06/swa_edits_9.16.pdf

Ghazisaeedi, F., Ciesinski, L., Bednorz, C., Johanns, V., Pieper, L., Tedin, K., Wieler, L. H., and Günther, S. (2020). Phenotypic zinc resistance does not correlate with antimicrobial multi-resistance in fecal *E. coli* isolates of piglets. *Gut Pathogens*, 12(1), 4. https://doi.org/10.1186/s13099-019-0342-5

Glover, B., Wentzel, J., Jenkins, A., and Van Vuuren, M. (2017). The first report of *Escherichia fergusonii* isolated from non-human primates, in Africa. *One Health,* 3(June), 70-75. https://doi.org/10.1016/j.onehlt.2017.05.001

Gouws, J. (2015). Colistin use by veterinarians. *Official Newsletter of the South African Veterinary Council*. Available at: internal-pdf://254.139.190.238/RSA_Use_of_Colistin_SAVC_and_MCC.pdf

Greenberg, J., Seebach, R., Luhmann, A., Zylstra, D., and Wentz, J. (2007). Co-operative Sanitation Project Pellsrus Township South Africa. *Waterlines*, 26(2), 18–20.

Guerra, B., Junker, E., Schroeter, A., Malorny, B., Lehmann, S., and Helmuth, R. (2003). Phenotypic and genotypic characterization of antimicrobial resistance in German *Escherichia coli* isolates from cattle, swine and poultry. *Journal of Antimicrobial Chemotherapy*, 52(3), 489–492.

Guo, J., Li, J., Chen, H., Bond, P. L., and Yuan, Z. (2017). Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic resistance genes and mobile genetic elements. *Water Research*, 123, 468–478. https://doi.org/https://doi.org/10.1016/j.watres.2017.07.002

Guo, M. T., Yuan, Q. Bin, and Yang, J. (2015). Distinguishing effects of ultraviolet exposure and chlorination on the horizontal transfer of antibiotic resistance genes in municipal wastewater. *Environmental Science and Technology*, 49(9), 5771–5778. https://doi.org/10.1021/acs.est.5b00644

Guo, Y., Xun, M., and Han, J. (2018). A bovine myeloid antimicrobial peptide (BMAP-28) and its analogs kill pan-drug-resistant *Acinetobacter baumannii* by interacting with outer

membrane protein A (OmpA). *Medicine*, 97(42). https://doi.org/10.1097/MD.0000000000012832

Gurbanov, R., and Severcan, F. (2020). Bacterial biofilms in bioremediation of metal-contaminated aquatic environments. In *Methods for Bioremediation of Water and Wastewater Pollution*. 117–135. https://doi.org/10.1007/978-3-030-48985-4_5

Hallatschek, O., Hersen, P., Ramanathan, S., and Nelson, D. R. (2007). Genetic drift at expanding frontiers promotes gene segregation. *National Academy of Sciences*, 104(50), 19926–19930. https://doi.org/10.1073/pnas.0710150104

Harrison, J. J., Ceri, H., and Turner, R. J. (2007). Multimetal resistance and tolerance in microbial biofilms. *Nature Reviews Microbiology*, 5(12), 928-38. doi: 10.1038/nrmicro1774. PMID: 17940533.https://doi.org/10.1038/nrmicro1774

Harrison, J. J., Ceri, H., Stremick, C. A., and Turner, R. J. (2004). Biofilm susceptibility to metal toxicity. *Environmental Microbiology*, 6(12), 1220–1227. https://doi.org/10.1111/j.1462-2920.2004.00656.x

Harrison, J. J., Turner, R. J., and Ceri, H. (2005). Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic *Pseudomonas aeruginosa*. *Environmental Microbiology*, 7(7), 981–994. https://doi.org/10.1111/j.1462-2920.2005.00777.x

Harvey, E. (2018). Township sanitation remains the pits. *The Mail and Guardian.* Available at: https://mg.co.za/article/2018-12-07-00-township-sanitation-remains-the-pits/

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E. T., and Schwartz, T. (2017). Occurrence of the *mcr-1* colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany. *Frontiers in Microbiology*, 8. https://doi.org/10.3389/fmicb.2017.01282

Heo, J. M., Opapeju, F. O., Pluske, J. R., Kim, J. C., Hampson, D. J., and Nyachoti, C. M. (2013). Gastrointestinal health and function in weaned pigs: A review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. *Journal of Animal Physiology and Animal Nutrition*, 97(2), 207–237. https://doi.org/10.1111/j.1439-0396.2012.01284.x

Herbig, F. J. W. (2019). Talking dirty - effluent and sewage irreverence in South Africa: A conservation crime perspective. *Cogent Social Sciences*, 5(1). https://doi.org/10.1080/23311886.2019.1701359

Heuer, H., and Smalla, K. (2007). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environmental Microbiology*, 9(3), 657–666. https://doi.org/10.1111/j.1462-2920.2006.01185.x

Heuer, H., Focks, A., Lamshöft, M., Smalla, K., Matthies, M., Spiteller, M., ft, M. L., Smalla, K., Matthies, M., and Spiteller, M. (2008). Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. *Soil Biology and Biochemistry*, 40(7), 1892–1900. https://doi.org/10.1016/j.soilbio.2008.03.014

Hölzel, C. S., Müller, C., Harms, K. S., Mikolajewski, S., Schäfer, S., Schwaiger, K., and Bauer, J. (2012). Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. *Environmental Research*, 113, 21–27. https://doi.org/10.1016/j.envres.2012.01.002

Hu, Y., Liu, F., Lin, I. Y., Gao, G. F., Zhu, B., Arcilla, M. S., Hattem, J. M., Matamoros, S., Melles, D. C., Penders, J., Jong, M. D., Tse, H., Yuen, K. Y., Olaitan, A. O., Chabou, S., Okdah, L., Morand, S., Rolain, J. M., Webb, H. E., Granier, S A., Marault, M., Millemann, Y., Bakker, H. C., and Nightingale, K. K. (2016). Dissemination of the *mcr-1* colistin resistance gene. *Lancet Infectious Diseases*, 16. https://doi.org/10.1016/S1473-3099(15)00538-1

Hua, M., Huang, W., Chen, A., Rehmet, M., Jin, C., and Hua, Z. (2020). Comparison of Antimicrobial Resistance Detected in Environmental and Clinical Isolates from Historical Data for the US. BioMed Research International. https://doi.org/10.1155/2020/4254530

Huang, C.-H., Renew, J. E., Smeby, K. L., Pinkston, K., and Sedlak, D. L. (2011). Assessment of potential antibiotic contaminants in water and preliminary occurrence analysis. *Journal of Contemporary Water Research and Education*, 120(1), 4. internal-pdf://137.87.178.222/Assessment of Potential Antibiotic Contaminant.pdf

Hughes, D., and Andersson, D. I. (2017). Environmental and genetic modulation of the phenotypic expression of antibiotic resistance. *FEMS Microbiology Reviews*, 004, 374–391. https://doi.org/10.1093/femsre/fux004

Hultman, J., Tamminen, M., Pärnänen, K., Cairns, J., Karkman, A., and Virta, M. (2018). Host range of antibiotic resistance genes in wastewater treatment plant influent and effluent. *FEMS Microbiology Ecology*, 94(4), fiy038–fiy038. https://doi.org/10.1093/femsec/fiy038

Ismail, A. (2016). The most important thing South Africans should know about superbugs. Colds and Flu. *Health 24*. Available at: https://www.health24.com/Medical/Flu/News/the-most-important-thing-south-africans-should-know-about-superbugs-20160531

Iweriebor, B., Gaqavu, S., Obi, L., Nwodo, U., and Okoh, A. (2015). Antibiotic Susceptibilities of Enterococcus Species Isolated from Hospital and Domestic Wastewater Effluents in Alice, Eastern Cape Province of South Africa. *International Journal of Environmental Research and Public Health*, 12(4), 4231. http://www.mdpi.com/1660-4601/12/4/4231

Jackson, L. M. D., Kroukamp, O., and Wolfaardt, G. M. (2015). Effect of carbon on whole-biofilm metabolic response to high doses of streptomycin. *Frontiers in Microbiology*, 6, 953. https://doi.org/10.3389/fmicb.2015.00953

Jackson, L. M. D., Kroukamp, O., Yeung, W. C., Ronan, E., Liss, S. N., and Wolfaardt, G. M. (2019). Species interaction and selective carbon addition during antibiotic exposure enhances bacterial survival. *Frontiers in Microbiology*, 10, 2730. https://doi.org/10.3389/fmicb.2019.02730

Janda, J. M., and Abbott, S. L. (2010). The Genus *Aeromonas*: Taxonomy, pathogenicity, and infection. *Clinical Microbiology Reviews*, 23(1), 35–73. https://doi.org/10.1128/cmr.00039-09

Jasim, N. A. (2020). The design for wastewater treatment plant (WWTP) with GPS X modelling. *Cogent Engineering*, 7(1). https://doi.org/10.1080/23311916.2020.1723782

Johanns, V. C., Ghazisaeedi, F., Epping, L., Semmler, T., Lübke-Becker, A., Pfeifer, Y., Bethe, A., Eichhorn, I., Merle, R., Walther, B., and Wieler, L. H. (2019). Effects of a four-week high-dosage zinc oxide supplemented diet on commensal *Escherichia coli* of weaned pigs. *Frontiers in Microbiology*, 10, 2734. https://doi.org/10.3389/fmicb.2019.02734

Kao, C., Lin, X., Yi, G., Zhang, Y., Rowe-Magnus, D. A., and Bush, K. (2016). Cathelicidin antimicrobial peptides with reduced activation of toll-like receptor signaling have potent bactericidal activity against colistin-resistant bacteria. *MBio*, 7(5). https://doi.org/10.1128/mBio.01418-16

Karlamangla, S. (2017). Patient had *E. coli* superbug. *Los Angeles Times.* Available at: https://www.latimes.com/local/california/la-me-ln-colistin-resistance-20170131-story.html

Kasprzyk-Hordern, B., Dinsdale, R. M., and Guwy, A. J. (2009). The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. *Water Research*, 43(2), 363–380.

Kaur, S. P., Rao, R., and Nanda, S. (2011). Amoxicillin: a broad-spectrum antibiotic. *International Journal of Pharmacy and Pharmaceutical Sciences*, 3(3), 30–37. https://innovareacademics.in/journal/ijpps/Vol3Issue3/2249.pdf

Knapp, C. W., McCluskey, S. M., Singh, B. K., Campbell, C. D., Hudson, G., and Graham, D. W. (2011). Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived Scottish soils. *PloS One*, 6(11), e27300. https://doi.org/10.1371/journal.pone.0027300

Kneis, D., Berendonk, T. U., and Heß, S. (2019). High prevalence of colistin resistance genes in German municipal wastewater. *Science of the Total Environment*, 694, 133454. https://doi.org/10.1016/j.scitotenv.2019.07.260

Knobler, S. L., Lemon, S. M., Najafi, M., and Burroughs, T. (2003). Factors contributing to the emergence of resistance. The Resistance Phenomenon in Microbes and Infectious Disease Vectors: Implications for Human Health and Strategies for Containment: Workshop Summary. *National Academies Press (US)*. https://www.ncbi.nlm.nih.gov/books/NBK97126/

Köck, R., Daniels-Haardt, I., Becker, K., Mellmann, A., Friedrich, A. W., Mevius, D., Schwarz, S., and Jurke, A. (2018). Carbapenem-resistant Enterobacteriaceae in wildlife, food-producing, and companion animals: a systematic review. *Clinical Microbiology and Infection*, 24(12), 1241–1250. https://doi.org/10.1016/j.cmi.2018.04.004

Kohanim, Y. K., Levi, D., Jona, G., Towbin, B. D., Bren, A., and Alon, U. (2018). A bacterial growth law out of steady state. *Cell Reports*, 23(10), 2891–2900. https://doi.org/10.1016/j.celrep.2018.05.007

Kraemer, S. A., Ramachandran, A., and Perron, G. G. (2019). Antibiotic pollution in the environment: From microbial ecology to public policy. *Microorganisms,* 7(6). https://doi.org/10.3390/microorganisms7060180

Kroukamp, O., Dumitrache, R. G., and Wolfaardt, G. M. (2010). Pronounced Effect of the Nature of the Inoculum on Biofilm Development in Flow Systems. *Applied and Environmental Microbiology,* 76(18), 6025-6031. https://doi.org/10.1128/AEM.00070-10

Kroukamp, O., and Wolfaardt, G. M. (2009). CO2 production as an indicator of biofilm metabolism. *Applied and Environmental Microbiology*, 75(13), 4391–4397. https://doi.org/10.1128/aem.01567-08

Kümmerer, K. (2009). Antibiotics in the aquatic environment – A review – Part II. *Chemosphere*, 75, 417–441. https://doi: 10.1016/j.chemosphere.2008.11.086., https://pdf.sciencedirectassets.com/271852/1-s2.0-S0045653509X00123/1-s2.0-S0045653508015099/main.pdf?X-Amz-Security-Token=IQoJb3JpZ2luX2VjELj%2F%2F%2F%2F%2F%2F%2F%2F%2F%2FwEaCXVzLWVhc3QtMSJHMEUCICzWaVCmDSRqzInclIRSushyJCey3%2BdkU0JlWmqAiNBAiEA59lRmng5U7

Labuschagne, Q., Schellack, N., Gous, A., Bronkhorst, E., Schellack, G., van Tonder, L., Truter, A., Smith, C., Lancaster, R., and Kolman, S. (2016). COLISTIN: adult and paediatric guideline for South Africa, 2016. *Southern African Journal of Infectious Diseases*, 31(1), 3–7. https://doi.org/10.1080/23120053.2016.1144285

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K. M., Wertheim, H. F. L., Sumpradit, N., Vlieghe, E., Hara, G. L., Gould, I. M., Goossens, H., Greko, C., So, A. D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A. Q., Qamar, F. N., Mir, F., Kariuki, S., Bhutta, Z. A., Coates, A., Bergstrom, R., Wright, G. D., Brown, E. D., and Cars, O. (2013). Antibiotic resistance—the need for global solutions. *The Lancet Infectious Diseases*, 13(12), 1057–1098. https://doi.org/10.1016/s1473-3099(13)70318-9

Lee, C.-Y., Lee, H.-F., Huang, F.-L., and Chen, P.-Y. (2009). Haemorrhagic bullae associated with a chicken scratch. *Annals of Tropical Paediatrics*, 29(4), 309–311.

Li, J., Shi, X., Yin, W., Wang, Y., Shen, Z., Ding, S., and Wang, S. (2017). A multiplex SYBR green Real-Time PCR assay for the detection of three colistin resistance genes from cultured bacteria, feces, and environment Samples. *Frontiers in Microbiology*, 8(October), 2078. https://doi.org/10.3389/fmicb.2017.02078

Lim, S. R., Lee, D.-H., Park, S. Y., Lee, S., Kim, H. Y., Lee, M.-S., Lee, J. R., Han, J. E., Kim, H. K., and Kim, J. H. (2019). Wild Nutria (*Myocastor coypus*) is a potential reservoir of

carbapenem-resistant and zoonotic *Aeromonas* spp. in Korea. *Microorganisms*, 7(8), 224. https://doi.org/10.3390/microorganisms7080224

Lin, J., Nishino, K., Roberts, M. C., Tolmasky, M., Aminov, R. I., and Zhang, L. (2015). Mechanisms of antibiotic resistance. *Frontiers in Microbiology*, 6, 34. https://doi.org/10.3389/fmicb.2015.00034

Lin, W., Li, S., Zhang, S., and Yu, X. (2016). Reduction in horizontal transfer of conjugative plasmid by UV irradiation and low-level chlorination. *Water Research*, 91, 331–338. https://doi.org/10.1016/j.watres.2016.01.020

Ling, L. L., Schneider, T., Peoples, A. J., Spoering, A. L., Engels, I., Conlon, B. P., Mueller, A., Schaberle, T. F., Hughes, D. E., Epstein, S., Jones, M., Lazarides, L., Steadman, V. A., Cohen, D. R., Felix, C. R., Fetterman, K. A., Millett, W. P., Nitti, A. G., Zullo, A. M., Chen, C., Lewis, K. (2015). A new antibiotic kills pathogens without detectable resistance. *Nature*, 517(7535), 455–459. https://doi.org/10.1038/nature14098

Liu, H., Zhu, J., Hu, Q., and Rao, X. (2016). *Morganella morganii*, a non-negligent opportunistic pathogen. *International Journal of Infectious Diseases*, 50, 10–17. https://doi.org/10.1016/j.ijid.2016.07.006

Liu, L., Lan, R., Liu, L., Wang, Y., Zhang, Y., Wang, Y., and Xu, J. (2017). Antimicrobial resistance and cytotoxicity of *Citrobacter* spp. in Maanshan Anhui province, China. *Frontiers in Microbiology*, 8(July), 1357. https://doi.org/10.3389/fmicb.2017.01357

Liu, Y.-Y., Wang, Y., Walsh, T. R., Yi, L.-X. X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., Huang, X., Yu, L.-F. F., Gu, D., Ren, H., Chen, X., Lv, L., He, D., Zhou, H., Liang, Z., Liu, J.-H. H., and Shen, J. (2016a). Emergence of plasmid-mediated colistin resistance mechanism *MCR-1* in animals and human beings in China: a microbiological and molecular biological study. *The Lancet Infectious Diseases*, 16(2), 161–168. https://doi.org/10.1016/S1473-3099(15)00424-7

Löffler, D., and Ternes, T. A. (2003). Analytical method for the determination of the aminoglycoside gentamicin in hospital wastewater via liquid chromatography-electrospray-tandem mass spectrometry. *Journal of Chromatography A*, 1000(1–2), 583–588. https://doi.org/10.1016/S0021-9673(03)00059-1

Lu, J., Breitwieser, F. P., Thielen, P., and Salzberg, S. L. (2017). Bracken: Estimating species abundance in metagenomics data. *PeerJ Computer Science*, 2017(1), e104. https://doi.org/10.7717/peerj-cs.104

Lupan, I., Carpa, R., Oltean, A., Kelemen, B. S., and Popescu, O. (2017). Release of antibiotic resistant bacteria by a waste treatment plant from Romania. *Microbes and Environments*, 32(3), 219–225. https://doi.org/10.1264/jsme2.ME17016

Madsen, J. S., Burmolle, M., Hansen, L. H., Sorensen, S. J., Burmølle, M., Hansen, L. H., and Sørensen, S. J. (2012). The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunology and Medical Microbiology*, 65(2), 183–195. https://doi.org/10.1111/j.1574-695X.2012.00960.x

Martínez, J. L. (2017). Effect of antibiotics on bacterial populations: A multi-hierachical selection process. *F1000Research,* 6. https://doi.org/10.12688/f1000research.9685.1

McIntosh, J. (2017). Everything you need to know about amoxicillin. 2018. Available at: https://www.medicalnewstoday.com/articles/158481.php#references

McKenna, M. (2013). Antibiotic resistance: The last resort. *Nature*, 499(7459), 394–396. https://doi.org/10.1038/499394a

Méric, G., Wick, R. R., Watts, S. C., Holt, K. E., and Inouye, M. (2019). Correcting index databases improves metagenomic studies. *bioRxiv*, 712166. https://doi.org/10.1101/712166

Mingeot-Leclercq, M.-P. P., Glupczynski, Y., and Tulkens, P. M. (1999). Aminoglycosides: activity and resistance. *Antimicrobial Agents and Chemotherapy*, 43(4), 727–737.

Minh, T. B., Leung, H. W., Loi, I. H., Chan, W. H., So, M. K., Mao, J. Q., Choi, D., Lam, J. C. W., Zheng, G., and Martin, M. (2009). Antibiotics in the Hong Kong metropolitan area: ubiquitous distribution and fate in Victoria Harbour. *Marine Pollution Bulletin*, 58(7), 1052–1062.

Miralles-Cuevas, S., Oller, I., Aguera, A., Llorca, M., Sanchez Perez, J. A., Malato, S., Agüera, A., Llorca, M., Sánchez Pérez, J. A., Malato, S., Aguera, A., Llorca, M., Sanchez Perez, J. A., and Malato, S. (2017). Combination of nanofiltration and ozonation for the remediation of real municipal wastewater effluents: Acute and chronic toxicity assessment. *Journal of Hazardous Materials*, 323(A), 442–451. https://doi.org/10.1016/j.jhazmat.2016.03.013

Mishra, S., Bharagava, R. N., More, N., Yadav, A., Zainith, S., Mani, S., and Chowdhary, P. (2019). Heavy metal contamination: An alarming threat to environment and human health. *Environmental Biotechnology: For Sustainable Future*, 103–125. https://doi.org/10.1007/978-981-10-7284-0_5

Munir, M., Wong, K., and Xagoraraki, I. (2011). Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Research*, 45(2), 681–693. https://doi.org/10.1016/j.watres.2010.08.033

Murray, G. E., Tobin, R. S., Junkins, B., and Kushner, D. J. (1984). Effect of chlorination on antibiotic resistance profiles of sewage-related bacteria. *Applied and Environmental Microbiology*, 48(1), 73–77. https://doi.org/10.1128/AEM.48.1.73-77.1984

Muziasari, W. I., Managaki, S., Pärnänen, K., Karkman, A., Lyra, C., Tamminen, M., Suzuki, S., and Virta, M. (2014). Sulphonamide and trimethoprim resistance genes persist in sediments at Baltic Sea aquaculture farms but are not detected in the surrounding environment. *PloS One*, 9(3), e92702. https://doi.org/10.1371/journal.pone.0092702

Nagwekar, P. R. (2014). Removal of organic matter from wastewater by activated sludge process-review. *International Journal of Science, Engineering and Technology Research*, 3(5), 1260–1263. http://ijsetr.org/wp-content/uploads/2014/05/IJSETR-VOL-3-ISSUE-5-1260-1263.pdf

Napier, B. A., Burd, E. M., Satola, S. W., Cagle, S. M., Ray, S. M., Mcgann, P., Pohl, J., Lesho, E. P., and Weiss, D. S. (2013). Clinical use of colistin induces cross-resistance to host antimicrobials in *Acinetobacter baumannii*. *MBio*, 4(3). https://doi.org/10.1128/mBio.00021-13

National Department of Health. (2012). Antimicrobial Resistance National Strategy Framework 2014-2024 (Issue May). Available at: https://www.jpiamr.eu/wp-content/uploads/2018/10/Antimicrobial-Resistance-National-Strategy-Framework-2014-2024_South-Africa.pdf

National Department of Health. (2016). A Practical Guide to Antimicrobial Stewardship in South Africa.

National Health Laboratory Service. (2013). Western cape academic hospitals antimicrobial recommendations 2013.

National Institute for Communicable Diseases. (2019). AMR Surveillance - Antimicrobial Susceptibility Testing (AST) in SA. https://mstrweb.nicd.ac.za/MicroStrategy/asp/Main.aspx

Newton-Foot, M., Snyman, Y., Maloba, M. R. B., and Whitelaw, A. C. (2017). Plasmid-mediated *mcr-1* colistin resistance in *Escherichia coli* and *Klebsiella* spp. clinical isolates from the Western Cape region of South Africa. *Antimicrobial Resistance and Infection Control*, 6(1), 78. https://doi.org/10.1186/s13756-017-0234-8

O'Neill, J. (2014). Antimicrobial resistance: tackling a crisis for the health and wealth of nations. Review on Antimicrobial Resistance. Available at: https://amr-review.org/Publications.html

Obayiuwana, A., Ogunjobi, A., Yang, M., and Ibekwe, M. (2018). Characterization of bacterial communities and their antibiotic resistance profiles in wastewaters obtained from pharmaceutical facilities in lagos and Ogun states, Nigeria. *International Journal of Environmental Research and Public Health*, 15(7). https://doi.org/10.3390/ijerph15071365

Olaitan, A. O., Morand, S., and Rolain, J. M. (2014). Mechanisms of polymyxin resistance: Acquired and intrinsic resistance in bacteria. *Frontiers in Microbiology*, 5(November). https://doi.org/10.3389/fmicb.2014.00643

Osunmakinde, C. O., Selvarajan, R., Mamba, B. B., and Msagati, T. A. M. (2019). Profiling bacterial diversity and potential pathogens in wastewater treatment plants using high-throughput sequencing analysis. *Microorganisms*, 7(11). https://doi.org/10.3390/microorganisms7110506

Ovejero, C. M., Delgado-Blas, J. F., Calero-Caceres, W., Muniesa, M., and Gonzalez-Zorn, B. (2017). Spread of *mcr-1*-carrying Enterobacteriaceae in sewage water from Spain. *Journal of Antimicrobial Chemotherapy*, 72(4), 1050–1053. https://doi.org/10.1093/jac/dkw533

Oz, T., Guvenek, A., Yildiz, S., Karaboga, E., Tamer, Y. T., Mumcuyan, N., Ozan, V. B., Senturk, G. H., Cokol, M., Yeh, P., and Toprak, E. (2014). Strength of selection pressure is an important parameter contributing to the complexity of antibiotic resistance evolution. *Molecular Biology and Evolution*, 31(9), 2387–2401. https://doi.org/10.1093/molbev/msu191

Palomo-Briones, R., Ovando-Franco, M., Razo-Flores, E., Celis, L. B., Rangel-Méndez, J. R., Vences-Álvarez, E., Cruz, R., Lázaro, I., and Briones-Gallardo, R. (2016). An overview of

reclaimed wastewater reuse in gold heap leaching. *Mineral Processing and Extractive Metallurgy Review*, 37(4), 274–285. https://doi.org/10.1080/08827508.2016.1190356

Papp-Wallace, K. M., Endimiani, A., Taracila, M. A., and Bonomo, R. A. (2011). Carbapenems: Past, present, and future. *Antimicrobial Agents and Chemotherapy*, 55(11), 4943–4960. https://doi.org/10.1128/AAC.00296-11

Pazda, M., Kumirska, J., Stepnowski, P., and Mulkiewicz, E. (2019). Antibiotic resistance genes identified in wastewater treatment plant systems – A review. *Science of the Total Environment*, 697, 134023). https://doi.org/10.1016/j.scitotenv.2019.134023

Peng, X., Tan, J., Tang, C., Yu, Y., and Wang, Z. (2008). Multiresidue determination of fluoroquinolone, sulfonamide, trimethoprim, and chloramphenicol antibiotics in urban waters in China. *Environmental Toxicology and Chemistry*, 27(1), 73–79. https://doi.org/10.1897/06-650.1

Perez, F., and Van Duin, D. (2013). Carbapenem-resistant Enterobacteriaceae: A menace to our most vulnerable patients. *Cleveland Clinic Journal of Medicine*, 80(4), 225–233. https://doi.org/10.3949/ccjm.80a.12182

Pérez-Rodríguez, F., and Mercanoglu Taban, B. (2019). A state-of-art review on multi-drug resistant pathogens in foods of animal origin: Risk factors and mitigation strategies. *Frontiers in Microbiology*, 10, 2091. https://doi.org/10.3389/fmicb.2019.02091

Petrie, B., Barden, R., and Kasprzyk-Hordern, B. (2015). A review on emerging contaminants in wastewaters and the environment: current knowledge, understudied areas and recommendations for future monitoring. *Water Research*, 72(0), 3–27. https://doi.org/10.1016/j.watres.2014.08.053

Pillay, K. (2017). Warning of Rise of Superbugs. *The Mercury*.

Pruden, A., Arabi, M., and Storteboom, H. N. (2012). Correlation between upstream human activities and riverine antibiotic resistance genes. *Environmental Science and Technology*, 46(21), 11541–11549. https://doi.org/10.1021/es302657r

PubChem: ZnO. (n.d.). Zinc oxide | ZnO - PubChem. Retrieved January 5, 2021, from https://pubchem.ncbi.nlm.nih.gov/compound/14806

PubChem: $ZnCl_2$. (n.d.). Zinc chloride | $ZnCl_2$ - PubChem. Retrieved January 5, 2021, from https://pubchem.ncbi.nlm.nih.gov/compound/5727

Rahman, T., Yarnall, B., and Doyle, D. A. (2017). Efflux drug transporters at the forefront of antimicrobial resistance. *European Biophysics Journal*, 46(7), 647–653. https://doi.org/10.1007/s00249-017-1238-2

Read, A. F., and Woods, R. J. (2014). Antibiotic resistance management. *Evolution, Medicine, and Public Health*, 2014(1), 147. https://doi.org/10.1093/emph/eou024

Renew, J. E., Smeby, K. L., Pinkston, K., and Sedlak, D. L. (2001). Assessment of potential antibiotic contaminants in water and preliminary occurrence analysis. *Journal of Contemporary Water Research and Education*, 120(1), 30–40. https://doi.org/10.1142/9789812799555_0004

Rhouma, M., Beaudry, F., and Letellier, A. (2016). Resistance to colistin: what is the fate for this antibiotic in pig production? *International Journal of Antimicrobial Agents*, 48(2), 119–126. https://doi.org/10.1016/j.ijantimicag.2016.04.008

Rice, L. B. (2008). Federal funding for the study of antimicrobial resistance in nosocomial pathogens: No ESKAPE. *The Journal of Infectious Diseases*, 197(8), 1079–1081. https://doi.org/10.1086/533452

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., Michael, I., and Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Science of The Total Environment*, 447(May), 345–360. https://doi.org/https://doi.org/10.1016/j.scitotenv.2013.01.032

Rolain, J. M. (2013). Food and human gut as reservoirs of transferable antibiotic resistance encoding genes. *Frontiers in Microbiology*, 4(June), 173. https://doi.org/10.3389/fmicb.2013.00173

Rolain, J. M., Kempf, M., Leangapichart, T., Chabou, S., Olaitan, A. O., Le Page, S., Morand, S., and Raoult, D. (2016). Plasmid-mediated *mcr-1* gene in colistin-resistant clinical isolates of *Klebsiella pneumoniae* in France and Laos. *Antimicrobial Agents and Chemotherapy,* 60(11), 6994-6995. *American Society for Microbiology*. https://doi.org/10.1128/AAC.00960-16

Ruiz, I. G., Orruño, M., Garaizabal, I., Arana, I., and Barcina, I. (2013). Validation of a sonication-based method for bacterial dislodgement from flocs. *Journal of Microbiology Research*, 2013(6), 208–212. https://doi.org/10.5923/j.microbiology.20130306.03

Rumbaugh, E. (2014). Plug flow, complete mix which is better? *Biological Waste Expert*. Available at: https://www.biologicalwasteexpert.com/blog/plug-flow-complete-mix-which-is-better

Sabri, N. A., van Holst, S., Schmitt, H., van der Zaan, B. M., Gerritsen, H. W., Rijnaarts, H. H. M., and Langenhoff, A. A. M. (2020). Fate of antibiotics and antibiotic resistance genes during conventional and additional treatment technologies in wastewater treatment plants. *Science of the Total Environment*, 741. https://doi.org/10.1016/j.scitotenv.2020.140199

Sahoo, D., Bhatt, M., Jena, S., Dash, D., and Chayani, N. (2015). Study of biofilm in bacteria from water pipelines. *Journal of Clinical and Diagnostic Research*, 9(3), 9–11. https://doi.org/10.7860/JCDR/2015/12415.5715

Salkind, A. R., and Rao, K. C. (2011). Antibiotic prophylaxis to prevent surgical site infections. *American Family Physician*, 83(5). www.aafp.org/afpAmericanFamilyPhysician585

Samadi, N., Aali, R., Asgari, E., Mirhosaeini, H., Shahriari, A., and Mahmoodi, F. (2015). Identification of clinically antibiotic resistant genes *Aac (3)-IIa* and *Aac (6')-Ib* in wastewater samples by multiplex PCR. *Environmental Health Engineering and Management Journal,* 2(2), 47–52. http://ehemj.com/article-1-72-en.html

Sano, D., Amarasiri, M., Hata, A., Watanabe, T., and Katayama, H. (2016). Risk management of viral infectious diseases in wastewater reclamation and reuse: Review. *Environment International*, 91, 220-229. https://doi.org/10.1016/j.envint.2016.03.001

Schwartz, T., Kohnen, W., Jansen, B., and Obst, U. (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiology Ecology*, 43(3), 325–335. https://doi.org/https://doi.org/10.1016/S0168-6496(02)00444-0

Segata, N., Waldron, L., Ballarini, A., Narasimhan, V., Jousson, O., and Huttenhower, C. (2012). Metagenomic microbial community profiling using unique clade-specific marker genes. *Nature Methods*, 9(8), 811–814. https://doi.org/10.1038/nmeth.2066

Sehar, S., and Naz, I. (2016). Role of the biofilms in wastewater treatment. In Microbial Biofilms - Importance and Applications. *InTech Open*. Available at: https://doi.org/10.5772/63499

Seiler, C., and Berendonk, T. U. (2012). Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Frontiers in Microbiology,* 3(December). https://doi.org/10.3389/fmicb.2012.00399

Sharma, D., Misba, L., and Khan, A. U. (2019). Antibiotics versus biofilm: An emerging battleground in microbial communities. *Antimicrobial Resistance and Infection Control,* 8(1), 1-10. https://doi.org/10.1186/s13756-019-0533-3

Sibanda, E. L., Weller, I. V. D., Hakim, J. G., and Cowan, F. M. (2011). Does trimethoprim-sulfamethoxazole prophylaxis for HIV induce bacterial resistance to other antibiotic classes?: Results of a systematic review. *Clinical Infectious Diseases*, 52(9), 1184–1194. https://doi.org/10.1093/cid/cir067

Singer, A. C., Shaw, H., Rhodes, V., and Hart, A. (2016). Review of Antimicrobial Resistance in the Environment and Its Relevance to Environmental Regulators. *Frontiers in Microbiology*, 7(November), 1728. https://doi.org/10.3389/fmicb.2016.01728

Singh, R., Paul, D., and Jain, R. K. (2006). Biofilms: implications in bioremediation. *Trends in Microbiology*, 14(9), 389–397. https://doi.org/10.1016/j.tim.2006.07.001

Slabbert, E., Van Heerden, C. J., and Jacobs, K. (2010). Optimisation of automated ribosomal intergenic spacer analysis for the estimation of microbial diversity in fynbos soil. *South African Journal of Science*, 106(7–8). https://doi.org/10.4102/sajs.v106i7/8.329

Smilack, J. D. (1999). Trimethoprim-sulfamethoxazole. *Mayo Clinic Proceedings*, 74(7), 730–734. https://doi.org/10.4065/74.7.730

Sowby, R. (2014). The Urban Water Cycle: Sustaining Our Modern Cities. *National Geographic Society Newsroom*. Available at: https://blog.nationalgeographic.org/2014/03/19/the-urban-water-cycle-sustaining-our-modern-cities/

Stalder, T., and Top, E. (2016). Plasmid transfer in biofilms: A perspective on limitations and opportunities. *npj Biofilms and Microbiomes*, 2, 16022). https://doi.org/10.1038/npjbiofilms.2016.22

Stats SA. (2019). Statistical Release P0302: Mid-year population estimates. Available at: https://www.statssa.gov.za/publications/P0302/P03022019.pdf

Stepanauskas, R., Glenn, T. C., Jagoe, C. H., Tuckfield, R. C., Lindell, A. H., and McArthur, J. V. (2005). Elevated microbial tolerance to metals and antibiotics in metal-contaminated industrial environments. *Environmental Science and Technology*, 39(10), 3671–3678. https://doi.org/10.1021/es048468f

Stone, W., Tolmay, J., Tucker, K., and Wolfaardt, G. M. (2020). Disinfectant, soap or probiotic cleaning? Surface microbiome diversity and biofilm competitive exclusion. *Microorganisms*, 8(11), 1–19. https://doi.org/10.3390/microorganisms8111726

Suzuki, S, Ogo, M., Miller, T. W., Shimizu, A., Takada, H., and Siringan, M. A. (2013). Who possesses drug resistance genes in the aquatic environment?: sulfamethoxazole (SMX) resistance genes among the bacterial community in water environment of Metro-Manila, Philippines. *Frontiers in Microbiology*, 4, 102. https://doi.org/10.3389/fmicb.2013.00102

Suzuki, S., Ogo, M., Koike, T., Takada, H., and Newman, B. (2015). Sulfonamide and tetracycline resistance genes in total- and culturable-bacterial assemblages in South African aquatic environments. *Frontiers in Microbiology*, 6(796), 1–8. https://doi.org/10.3389/fmicb.2015.00796

Swann, M. M., Baxter, K. L., and Field, H. I. (1969). Report of the joint committee on the use of antibiotics in animal husbandry and veterinary medicine. HMSO, London.

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K.-H. H., Gützkow, T., Eichler, W., Pühler, A., and Schlüter, A. (2009). Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology*, 155(7), 2306–2319. https://doi.org/doi:10.1099/mic.0.028233-0

Tacão, M., Correia, A., and Henriques, I. S. (2015). Low prevalence of carbapenem-resistant bacteria in river water: Resistance is mostly related to intrinsic mechanisms. *Microbial Drug Resistance*, 21(5), 497–506. https://doi.org/10.1089/mdr.2015.0072

Tahrani, L., Van Loco, J., Ben Mansour, H., and Reyns, T. (2016). Occurrence of antibiotics in pharmaceutical industrial wastewater, wastewater treatment plant and sea waters in Tunisia. *Journal of Water and Health*, 14(2), 208. http://jwh.iwaponline.com/content/14/2/208.abstract

Tang, J., Bu, Y., Zhang, X.-X. X., Huang, K., He, X., Ye, L., Shan, Z., and Ren, H. (2016). Metagenomic analysis of bacterial community composition and antibiotic resistance genes in

a wastewater treatment plant and its receiving surface water. *Ecotoxicology and Environmental Safety*, 132, 260–269. https://doi.org/10.1016/j.ecoenv.2016.06.016

Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K., and Sutton, D. J. (2012). Heavy metal toxicity and the environment. *Experientia supplementum,* 101, 133–164. https://doi.org/10.1007/978-3-7643-8340-4_6

Turner, R. J. (2017). Metal-based antimicrobial strategies. *Microbial Biotechnology*, 10(5), 1062–1065. https://doi.org/10.1111/1751-7915.12785

Vadstein, O., Attramadal, K. J. K., Bakke, I., and Olsen, Y. (2018). K-Selection as microbial community management strategy: A method for improved viability of larvae in aquaculture. *Frontiers in Microbiology*, 9(November), 2730. https://doi.org/10.3389/fmicb.2018.02730

Van Boeckel, T. P., Glennon, E. E., Chen, D., Gilbert, M., Robinson, T. P., Grenfell, B. T., Levin, S. A., Bonhoeffer, S., and Laxminarayan, R. (2017). Reducing antimicrobial use in food animals. *Science*, 357(6358), 1350–1352. https://doi.org/10.1126/science.aao1495

van der Zee, A., Roorda, L., Bosman, G., Fluit, A. C., Hermans, M., Smits, P. H. M., van der Zanden, A. G. M., Te Witt, R., Bruijnesteijn van Coppenraet, L. E. S., Cohen Stuart, J., and Ossewaarde, J. M. (2014). Multi-centre evaluation of real-time multiplex PCR for detection of carbapenemase genes *OXA-48, VIM, IMP, NDM* and *KPC*. *BMC Infectious Diseases*, 14(1), 27. https://doi.org/10.1186/1471-2334-14-27

van Hoek, A., Mevius, D., Guerra, B., Mullany, P., Roberts, A. P., and Aarts, H. J. M. M. (2011). Acquired Antibiotic Resistance Genes: An Overview. *Frontiers in Microbiology*, 2(September), 203. https://doi.org/10.3389/fmicb.2011.00203

Ventola, C. L. (2015). The Antibiotic Resistance Crisis: Part 1: Causes and Threats. *Pharmacy and Therapeutics*, 40(4), 277–283. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4378521/

Visser, W. P. (2018). A perfect storm: The ramifications of Cape Town's drought crisis. *The Journal for Transdisciplinary Research in Southern Africa*, 14(1), 10. https://doi.org/10.4102/td.v14i1.567

Wade, W. (2002). Unculturable bacteria—the uncharacterized organisms that cause oral infections. *Journal of the Royal Society of Medicine*, 95(2), 81–83. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1279316/

Waso, M., Ndlovu, T., Dobrowsky, P. H., Khan, S., and Khan, W. (2016). Presence of microbial and chemical source tracking markers in roof-harvested rainwater and catchment systems for the detection of fecal contamination. *Environmental Science and Pollution Research*, 23(17), 16987–17001.

Watkinson, A. J., Murby, E. J., Kolpin, D. W., and Costanzo, S. D. (2009). The occurrence of antibiotics in an urban watershed: from wastewater to drinking water. *Science of the Total Environment*, 407(8), 2711–2723. https://doi.org/10.1016/j.scitotenv.2008.11.059

Wellington, E. M. H. H., Boxall, A. B. A. A., Cross, P., Feil, E. J., Gaze, W. H., Hawkey, P. M., Johnson-Rollings, A. S., Jones, D. L., Lee, N. M., Otten, W., Thomas, C. M., and Williams, A. P. (2013). The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *The Lancet Infectious Diseases*, 13(2), 155–165. https://doi.org/10.1016/s1473-3099(12)70317-1

Wininger, D. A., and Fass, R. J. (2002). Impact of trimethoprim-sulfamethoxazole prophylaxis on etiology and susceptibilities of pathogens causing human immunodeficiency virus-associated bacteremia. *Antimicrobial Agents and Chemotherapy*, 46(2), 594–597. https://doi.org/10.1128/AAC.46.2.594-597.2002

Wise, M. G., Estabrook, M. A., Sahm, D. F., Stone, G. G., and Kazmierczak, K. M. (2018). Prevalence of *mcr*-type genes among colistin-resistant Enterobacteriaceae collected in 2014-2016 as part of the INFORM global surveillance program. *PloS One*, 13(4), e0195281. https://doi.org/10.1371/journal.pone.0195281

Wood, D. E., Lu, J., and Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biology*, 20(1), 257. https://doi.org/10.1186/s13059-019-1891-0

Woodford, N., Wareham, D. W., Guerra, B., and Teale, C. (2013). Carbapenemase-producing Enterobacteriaceae and non-Enterobacteriaceae from animals and the environment: an emerging public health risk of our own making? *Journal of Antimicrobial Chemotherapy,* 69(2):287-91 https://doi.org/10.1093/jac/dkt392

World Health Organisation. (2011). Critically important antimicrobials for human medicine. WHO. Available at: https://apps.who.int/iris/bitstream/handle/10665/77376/9789241504485_eng.pdf?sequence=1

World Health Organisation. (2017). Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Available at: https://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf?ua=1

World Health Organisation. (2017a). One Health. Available at: https://www.who.int/news-room/q-a-detail/one-health

World Health Organisation. (n.d.). Electronic essential medicines list. Available at: https://list.essentialmeds.org/?section=338andindication=andyear=andage=andsex=

Wu, S., Dalsgaard, A., Hammerum, A. M., Porsbo, L. J., and Jensen, L. B. (2010). Prevalence and characterization of plasmids carrying sulfonamide resistance genes among *Escherichia coli* from pigs, pig carcasses and human. *Acta Veterinaria Scandinavica*, 52(1), 47. https://doi.org/10.1186/1751-0147-52-47

Xu, J., Xu, Y., Wang, H., Guo, C., Qiu, H., He, Y., Zhang, Y., Li, X., and Meng, W. (2015). Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river. *Chemosphere*, 119, 1379–1385. https://doi.org/10.1016/j.chemosphere.2014.02.040

Yang, F., Mao, D., Zhou, H., and Luo, Y. (2016). Prevalence and fate of carbapenemase genes in a wastewater treatment plant in Northern China. *PLoS One*, 11(5), e0156383. https://doi.org/10.1371/journal.pone.0156383

Yang, Y.-Q., Li, Y.-X., Lei, C.-W., Zhang, A.-Y., and Wang, H.-N. (2018). Novel plasmid-mediated colistin resistance gene *mcr-7.1* in *Klebsiella pneumoniae*. *Journal of Antimicrobial Chemotherapy*, 73, 1791–1795. https://doi.org/10.1093/jac/dky111

Yang, Ying, Li, B., Zou, S., Fang, H. H. P., and Zhang, T. (2014). Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. *Water Research*, 62, 97–106. https://doi.org/https://doi.org/10.1016/j.watres.2014.05.019

Yang, Yuyin, Dai, Y., Li, N., Li, B., Xie, S., and Liu, Y. (2017). Temporal and spatial dynamics of sediment anaerobic ammonium oxidation (Anammox) bacteria in freshwater lakes. *Microbial Ecology*, 73(2), 285–295. https://doi.org/10.1007/s00248-016-0872-z

Yazdankhah, S., Rudi, K., and Bernhoft, A. (2014). Zinc and copper in animal feed – development of resistance and co-resistance to antimicrobial agents in bacteria of animal

origin. *Microbial Ecology in Health and Disease*, 25(0). https://doi.org/10.3402/mehd.v25.25862

Ye, M. Q., Chen, G. J., and Du, Z. J. (2020). Effects of antibiotics on the bacterial community, metabolic functions and antibiotic resistance genes in mariculture sediments during enrichment culturing. *Journal of Marine Science and Engineering*, 8(8), 604. https://doi.org/10.3390/JMSE8080604

You, Y., and Silbergeld, E. K. (2014). Learning from agriculture: Understanding low-dose antimicrobials as drivers of resistome expansion. *Frontiers in Microbiology,* 5, (June), 284. https://doi.org/10.3389/fmicb.2014.00284

Zaidi, A. K., Huskins, W. C., Thaver, D., Bhutta, Z. A., Abbas, Z., and Goldmann, D. A. (2005). Hospital-acquired neonatal infections in developing countries. *Lancet*, 365(9465), 1175–1188. https://doi.org/10.1016/s0140-6736(05)71881-x

Zhang, G., Xia, T., Zhao, J., Liu, L., He, P., Zhang, S., and Zhang, L. (2020). Moderate tetrabasic zinc chloride supplementation improves growth performance and reduces diarrhea incidence in weaned pigs. *Asian-Australasian Journal of Animal Sciences*, 33(2), 264–276. https://doi.org/10.5713/ajas.18.0914

Zhang, T., and Li, B. (2011). Occurrence, transformation, and fate of antibiotics in municipal wastewater treatment plants. *Critical Reviews in Environmental Science and Technology*, 41(11), 951–998. https://doi.org/10.1080/10643380903392692

Zhao, Q., and Liu, Y. (2019). Is anaerobic digestion a reliable barrier for deactivation of pathogens in biosludge? *Science of the Total Environment*, 668, 893–902. https://doi.org/10.1016/j.scitotenv.2019.03.063